A biochemical system for rapid on/off switching of Ras activity

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List of Abbreviations

4E-BP	Eukaryotic translation initiation factor 4E binding protein 1
AP1	Activator protein 1
Bcl-2	B-cell lymphoma 2
CDK	Cyclin-dependent kinase
DAG	diacylglycerol
DAPI	4',6-Diamidino-2-phenylindole
DH domain	Dbl homology domain
DNA	Deoxyribonucleic acid
EdU	5-ethynyl-2’-deoxyuridine
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
eIF4E	Eukaryotic translation initiation factor 4E
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinase
FACS	Fluorescence-Activated Cell Sorting
FKBP12	FK506 binding protein of 12 kDa
FOXO	Forkhead transcription factor
FRB	FK506-Rapamycin-Binding
FTI	Farnesyltransferase inhibitor
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GGTI	Geranylgeranyltransferase inhibitor
GRB2	Growth-factor-receptor-bound protein 2
GSK-3β	Glycogen synthase kinase-3β
GTP	Guanosine 5’-triphosphate

HCC	Hepatocellular carcinoma
HPV	Human papillomavirus
H-Ras	Harvey murine sarcoma viruses
HVR	Hypervariable region
iDim	Heterodimerizer
K-Ras	Kirsten murine sarcoma viruses
KSR1	Kinase suppressor of Ras
MAPK	Mitogen-activated protein kinase
Mdm2	Murine double minute 2
MEF	Mouse embryonic fibroblast
MEK	Mitogen-activated protein kinase kinase
Mnk	MAPK-interacting kinases
MP1	MEK1 scaffolding protein
mRNA	Messenger RNA
mTORC	Mammalian target of rapamycin complex
NF1	Neurofibromin type 1
NF-kB	Nuclear factor-kB
N-Ras	Neuroblastoma sarcoma viruses
ODNs	Antisense oligonucleotides
PDK1	Phosphoinositide-dependent kinase 1
PH domain	Pleckstrin homology domain
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
pRB	Retinoblastoma protein
Ral	Ras like
RalGDS	Ral guanine nucleotide dissociation stimulator
Rap1	Ras-proximate-1
RAPTOR	Regulatory-associated protein of mTOR
Ras	Rat sarcoma
RBD	Ras binding domain

RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNAi	RNA interference
RTK	Receptor tyrosine kinase
S6K	S6 kinase
SD	Standard deviation
SH domain	Src homology domain
shRNA	Small hairpin RNA
Skp2	S-phase kinase-associated protein 2
SOS	Son of sevenless
STAT	Signal transducer and activator of transcription 3
TSC2	Tuberous sclerosis complex 2

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Summary

Ras is a monomeric membrane-associated GTP-binding protein that fluctuates between two distinct conformational states defined as active (GTP-loaded) or inactive (GDP-loaded). Ras protein is widely considered a critical pro-mitogenic signalling molecule with an essential role in cell cycle progression. This notion is based on multiple studies documenting that interfering with Ras activation in cells emerging from quiescence in response to growth factor stimulation prevents cell cycle entry and/or progression. This cell cycle blockade correlates with a drop in CyclinD levels (one of the key drivers of G1-to-S transition). Furthermore, Ras activation not only during G1 but also specifically during G2 phase in continuously cycling cells has been reported. Thus, it is assumed that Ras is engaged to function at multiple preordained stages through the cell cycle, arguably for phase-transition as well as cell fate decision-making. However, the precise role and importance of Ras-dependent signalling at each of those stages could not be proven to date, owing to the lack of experimental approaches for the acute inhibition of Ras activity.

We have developed a novel biochemical tool that enables direct, acute interference with Ras activity at any desired time-point of the cell cycle. The system consists of two protein partners: an anchor unit (FKBP-mCherry-K-Ras-hvr) confined to the cellular membrane and an effector unit coupled to the catalytic domain of the Ras-GAP NF1. The latter is recruited to the plasma membrane upon addition of the small molecule heterodimerizer, rendering it close to membrane-bound Ras. Biochemical analysis proved that EGF-dependent activation of Ras and its downstream effector Erk was fully abrogated by short-term (15 min) pre-treatment with the heterodimerizer. By examining the activity of other small GTPase family member like Rap1, largely unaffected in presence of the heterodimerizer, we provide evidence that the heterodimerization system is rather accurate and selective in inhibiting Ras alone. This statement is also supported by the analysis performed in wild type cells and those transduced with one of the components of the heterodimerization system. Using this approach to interrogate the time periods and effectors engaged by Ras throughout G1 confirmed a critical role for Ras in cell cycle entry of growth factor-stimulated quiescent cells

and G1/S transition through the regulation of cyclinD1 expression. Thus, in T98G cells emerging from quiescence, progressive ablation of Ras function throughout G1 phase revealed a necessity for mitogenic Ras signalling deep into G1, up to 5 hours after growth factor stimulation. In parallel, flow cytometric cell cycle profiling of asynchronous, actively cycling cells showed a G0-G1 arrest in the lack of Ras activity. Further analysis in continuously proliferating T98G cells indicated that the absence of Ras function in G2 and early G1 phase led to a decrease in the ensuing S phase entry accompanied by a reduction of cyclinD1 expression. Dissection of signalling downstream of Ras identified Erk and excluded PI3K/Akt and Ral as the mediator of its signals to the cell cycle machinery.

In conclusion, we have charted the temporal signalling program of Ras during G0/G1/S phase progression employing a novel experimental methodology for the acute and specific inhibition of Ras that may have broad applicability in many areas of signal transduction and molecular oncology research.

Zusammenfassung

Ras ist ein, als Monomer vorliegendes, Membran-assoziiertes GTP-Bindeprotein, welches zwei verschiedene Konfirmationen annehmen kann, welche als aktive (GTP-gebunden) und inaktive (GDP-gebunden) Form definiert sind. Das Ras-Protein wird als entscheidendes pro-mitotisches Signalmolekül, mit einer essentiellen Rolle im Zell-Zyklus-Fortschritt, angesehen. So wurde in zahlreichen Studien gezeigt, dass eine Störung der Ras-Aktivierung in Zellen, welche durch die Stimulation mit Wachstumsfaktoren aus dem Ruhestadium erweckt wurden, den Eintritt bzw. das Voranschreiten des Zellzyklus verhindert. Diese Blockade des Zellzyklus geht einher mit einem Abfall des Cyclin-D-Spiegels (ein Schlüssel-Faktor im Übergang von G1 zu S). Des Weiteren wurde eine Ras-Aktivität nicht nur während der G1-Phase, sondern speziell auch während der G2-Phase nachgewiesen, wenn die Zellen kontinuierlich den Zellzyklus durchlaufen. Daher wird vermutet, dass Ras in vielen Phasen des Zellzyklus eine wichtige Rolle spielt, besonders beim Übergang der einzelnen Phasen, sowie bei der Entscheidung über das Schicksal der Zelle. Die genaue Rolle und Bedeutung des Ras-abhängigen Signalweges während der einzelnen Phasen konnte bis heute jedoch noch nicht beschrieben werden, da experimentelle Wege, die Ras Aktivität unmittelbar zu inhibieren, fehlen.

Wir haben ein neues biochemisches Werkzeug entwickelt, welche es uns ermöglicht die Ras-Aktivität direkt und unmittelbar zu jedem gewünschten Zeitpunkt des Zellzyklus zu stören. Das System besteht aus zwei Protein-Partnern, einer Anker-Einheit (FKBP-mCherry-K—Ras-hvr), welche an die Zellmembran gebunden ist und einer Effektor-Einheit, welche mit der katalytischen Domäne von Ras-GAP NF1 verbunden ist. Letztere wird nach Zufügen des niedermolekularen Heterodimerisierer an die Plasmamembran rekrutiert, in die Nähe des Membran-gebundenen Ras. Biochemische Analysen haben gezeigt, dass kurzzeitige Vorbehandlungen (15 min) mit dem Heterodimerisierer die EGF-abhängige Aktivierung von Ras und dessen nachfolgendem Effektor Erk vollständig aufheben können. Durch die Untersuchung anderer Mitglieder der Familie der kleinen GTPasen, wie Rap1, welches von der Anwesenheit des Heterodimerisierers weitestgehend unaffektiert blieb, konnten wir

zeigen, dass das Heterodimerisierer-System recht präzise und selektiv ausschließlich Ras inhibiert. Diese Beobachtung wird durch Analysen in wild typ Zellen und Zellen, die nur mit einem Bestandteil des Heterodimerisierer-Systems transduziert wurden, unterstützt. Mit Hilfe dieser Vorgehensweise konnten die Ras-anhängigen Zeiträume und Effektoren während der G1 Phase untersucht werden. Dabei zeigte sich, dass Ras eine wichtige Rolle einnimmt, wenn Zellen aus dem Ruhezustand nach Wachstumsfaktor-Stimulation in den Zellzyklus eintreten, sowie beim Übergang der G1-Phase zur S-Phase, durch die Regulierung der CyclinD1-Expression. Durch die schrittweise Blockade der Ras-Funktion zu unterschiedlichen Zeitpunkten während der G1 Phase in T98G Zellen, welche aus dem Ruhezustand kommen, konnte die Notwendigkeit für die mitogene Ras-Signaltransduktion, für bis zu 5 Stunden nach der Stimulation mit Wachstums-Faktoren, nachgewiesen werden. Zusätzlich zeigte die Zellzyklus-Analyse asynchroner, aktiv zirkulierender Zellen mittels Durchflusszytometrie einen G0-G1-Arrest, wenn Ras nicht aktiv ist. Weitere Analysen in kontinuierlich teilenden T98G Zellen weist darauf hin, dass eine fehlende Ras-Funktion in der G2- und der frühen G1-Phase zu einem verminderten Eintritt in die S-Phase führt, begleitet von einem Rückgang der CyclinD1-Expression. Untersuchungen der Signalübertragung nachfolgend auf Ras zeigten, dass Erk und nicht PI3K/Akt der Mediator der Signaltransduktion durch Ras in der Zellzyklus-Maschinerie ist.

Zusammenfassend konnten wir, mit Hilfe einer neuen experimentellen Methode für die sofortige und spezifische Inhibition von Ras, einen zeitlichen Verlauf des Ras-Signalweg während des Voranschreitens der G0-, G1- und S-Phase skizzieren, welcher eine breite Anwendbarkeit in verschiedensten Bereichen der Signaltransduktion und molekularen onkologischen Forschung haben kann.

1. Introduction

1.1. Small G-proteins

G proteins, guanine nucleotide binding proteins, form a large superfamily and are involved in many signal transduction cascades. According to the molecular structure they can be divided into two big families: the heterotrimeric and monomeric G proteins.

The heterotrimeric G proteins are composed of three different subunits: alpha, beta and gamma. They transmit signals from the plasma membrane receptors to intracellular proteins affecting several signalling pathways, vesical trafficking and protein synthesis.

The monomeric G proteins are characterized by a low molecular weight that vary between 20 and 30 kDa. Because to their size they are usually referred to as small GPT-binding proteins. They are further classified into five subfamilies: Ras, Rho, Rab, Arf and Ran (Bourne, Sanders *et al.* 1990). Despite the structural similarities, each subfamily has a specific intracellular function. Ras proteins (collectively referring to N-, H- and K-Ras) are mainly involved in cell proliferation and gene expression. The Rho proteins control cytoskeletal reorganization as well as gene expression. Rab together with Arf proteins cooperate to regulate the traffic of vesicles while the Ran subfamily is necessary in nucleocytoplasmic transport and microtubule organisation during cell cycle progression (Matozaki, Nakanishi *et al.* 2000).

Small G proteins have a conserved amino acid sequence and therefore show a 30-55% homology (Hall 1990, Valencia, Chardin *et al.* 1991). These conserved regions are localized in the GDP/GTP-binding site (so-called G-domain) of the protein. All members of this family have a specific region responsible for the interaction with their downstream effectors. Small G proteins, but not all, undergo post-translational modifications in their COOH- or NH_2-termini (Moss and Vaughan 1995). Their intracellular localization is dependent on these post-translational modifications that determine if proteins will be present at the plasma membrane or other membrane compartments. Most of the GTP-binding proteins are found at the cytosolic side of the different membranes, whereas Ran is the only one that can be localized in the cytosol or in the nucleus (Takai, Sasaki *et al.* 2001).

1.2. Ras protein, a molecular switch

Ras proteins have an intrinsic ability to continuously fluctuate between two distinct conformational states depending on whether they are bound to GDP or GTP (Guanosine diphosphate and Guanosine-5'-triphosphate, respectively). Ras is activated upon GTP binding (active state) and can interact with its downstream effectors until hydrolysis of GTP to GDP occurs, leading to an inactive GDP-bound Ras (Fig.1A). Both GDP and GTP bind to the effector lobe (G domain) that is composed of five highly conserved loops. Binding of GTP is ensured by the interaction between three loops G1, G4 and G5, that induce the conformational change in the remaining loops G2 and G3, known as Switch I and II regions (Fig.1B). This new orientation of the protein facilitates the GTPase process by exposing the catalytic Mg^{2+} and reactive water molecules (Vetter and Wittinghofer 2001, Wittinghofer 2006, Bos, Rehmann *et al.* 2007).

All GTP-binding proteins have the same basic structure composed of six β-sheets and five α-helixes. However, the nucleotide-binding site exposes a sequence of 4 or 5 amino acids that are highly conserved. There are two essential motifs that mediate the nucleotide binding, the N/TKXXD motif and the GXXXXGKS/T motif (Saraste, Sibbald *et al.* 1990). The latter includes the conserved phosphate-binding loop (P loop). Moreover, an Asp side chain in the DXXG motif and Ala^{146} of the SAK motif ensures the high specificity for guanine nucleotides.

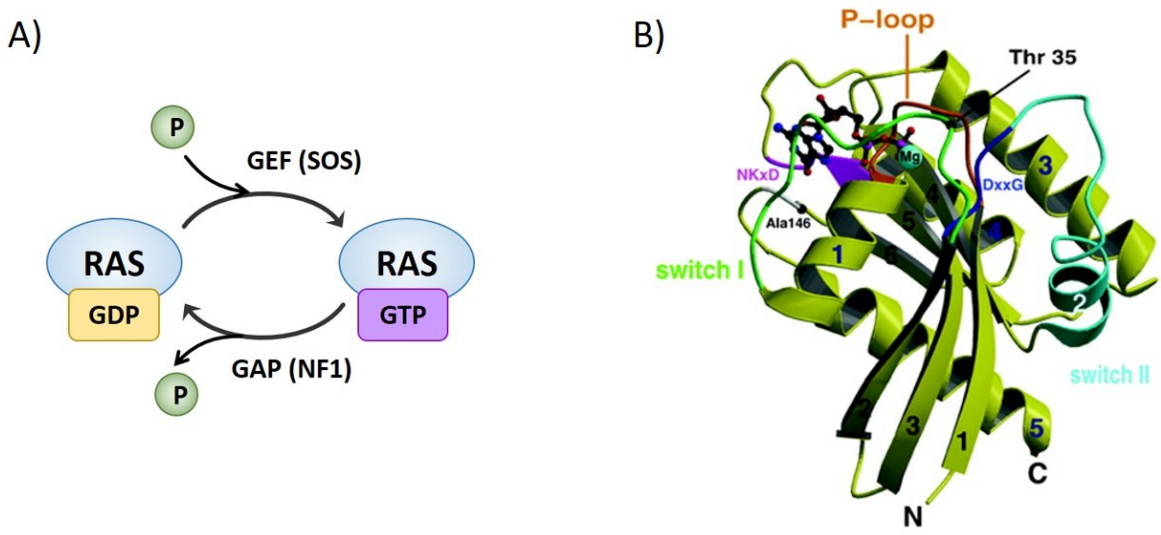

Figure 1. Ras, a small GTPase protein.

A) Simplified scheme of Ras cycling between the GTP- and GDP-bound forms. B) General structure of a guanine nucleotide binding protein. Image is taken from (Vetter and Wittinghofer 2001).

Once GTP is bound, the γ-phosphate oxygen creates 2 hydrogen bonds with two specific residues localised at the 35th position in Switch I and at the 60th position in Switch II, Threonine and Glycine respectively. The latter also binds Mg^{2+} ions by its side chain. Hydrolysis of the phosphate group induces relaxation of the Switch regions (Vetter and Wittinghofer 2001).

1.3. Ras, a monomeric G protein

Ras is the best characterized protein among the small G proteins. Its discovery dates back to the 1960s when viral genes with the capacity to transform cells in culture were identified. The nomenclature 'RAS' stems from the ability of these genes to induce *sarcomas in rats,* and its isoforms denominated by prefixes based on the discoverers' name, as H-Ras and K-Ras for Harvey and Kirsten murine sarcoma viruses, respectively (Der, Krontiris *et al.* 1982, Parada, Tabin *et al.* 1982, Santos, Tronick *et al.* 1982). More than 20 years later, other research groups observed the ability of these RAS genes to transform mouse fibroblasts (NIH3T3). Further studies identified a single missense mutation in codon 12 as the mechanism that conferred oncogenic potential to H-RAS and K-RAS genes. The occurrence of this point mutation at a high frequency in lung and colon cancer cells was soon documented (Reddy, Reynolds *et al.* 1982, Tabin, Bradley *et al.* 1982, Taparowsky, Suard *et al.* 1982, Capon, Seeburg *et al.* 1983). The third human RAS gene was first discovered in neuroblastoma lines after which it was appropriately named N-Ras (Hall, Marshall *et al.* 1983, Shimizu, Goldfarb *et al.* 1983). The discovery of oncogenic Ras spun-off a completely new research area towards the understanding of cancer at the molecular level. Further use of the NIH3T3 cells and DNA sequencing method led to the discovery of human Ras genes in pancreatic cancers in addition to the lung and colon cancers (Cox and Der 2010).

1.4. Ras isoforms

There are four different Ras proteins encoded from only three human RAS genes that have similar biochemical properties, structure and 82-90% sequence identity. The four isoforms are N-Ras, H-Ras and two alternative RNA splice variants of K-Ras resulting in two distinct proteins: K-Ras4A and K-Ras4B.

The human RAS gene product is a 188-189 amino acid protein consisting of three functionally significant regions: the effector lobe, the allosteric lobe and the hypervariable region. The effector lobe, the first 86 amino acids of the N-terminus, is the most conserved region of the protein showing a 100% sequence identity between all four proteins (Buhrman, O'Connor *et al.* 2011). This identity emphasized the importance of the region as it comprises the site where the exchange between GDP and GTP takes place, also called G domain. In addition, part of the G domain are the two switch regions, Switch I and Switch II. Their physical conformation depends on whether GTP or GDP is bound to Ras. The protein sequence continues with the allosteric lobe (87-166 residues) (Hobbs, Der *et al.* 2016). Ras isoforms share a 78-82% sequence homology in this region that is thought to be important for the membrane orientation of Switch II (Parker and Mattos 2015). C-terminal hypervariable region, as suggested by the name, is the least conserved between the isoforms with only 4% similarity. Together with the allosteric lobe they mediate proper localization and orientation of Ras on the plasma membrane, especially of the G domain for specific downstream effectors (Abankwa, Gorfe *et al.* 2008, Abankwa, Gorfe *et al.* 2010).

1.5. Post-translational modifications

It is well accepted that Ras can be biologically active only after being anchored to the plasma membrane. Over the last decades, many studies described a general mechanism on how newly synthesized Ras proteins undergo a number of post-translational modifications to ensure their translocation and association to the plasma membrane (Fig. 2). Main target for Ras post-translational processing are the C-terminal 24-25 residues that include the hypervariable region and the CAAX-box, emphasizing the major differences between Ras isoforms. Each of them is target for a specific modification. The CAAX box is a tetrapeptide motif that consists of one cysteine residue (C), two aliphatic amino acids (A) and a last nonspecific amino acid (Ahearn, Haigis *et al.* 2011, Hobbs, Der *et al.* 2016).

Like many other proteins Ras is synthesized in the cytoplasm. The first modification involves the covalent addition of a 15-carbon farnesyl isoprenoid lipid on the cysteine residue of the CAAX box catalysed by farnesyltransferase (FTase) (Casey, Solski *et al.* 1989, Hancock, Magee *et al.* 1989, Schafer, Kim *et al.* 1989). All Ras isoforms can undergo this type of modification, but in absence or inhibition of the FTase activity Ras is retained in the cytosol and therefore

not active. As a fail-safe mechanism, K- and N-Ras can be geranylgeranylated which allows further post-translational processing of the protein (Downward 2003). As a result an alternative C20 geranylgeranyl lipid is added to the protein. A proteolytic cleavage of the AAX peptide sequence followed by a carboxymethylation of the already farnesylated cysteine residue completes the modifications of the CAAX box. The last alterations occur in the endoplasmic reticulum (ER) and increase the hydrophobic nature of the C-terminus facilitating Ras association to the plasma membrane (Hancock, Cadwallader *et al.* 1991).

Moreover, a second membrane targeting element is required and is in fact provided by specific sequences in the hypervariable region (hvr). Except for K-Ras4B, Ras proteins are covalently modified at their hvr cysteine residues by Golgi-associated palmitoyltransferases. These enzymes catalyse the addition of a single palmitic acid in case of N-RAS and two separate palmitoylation modification for H-Ras (Hancock, Paterson *et al.* 1990). Proteins are further transported through the Golgi apparatus to the plasma membrane. K-Ras4B instead has a polylysine sequence (175-180 residues) in its hvr with a positive charge that facilitates the interaction with the negatively charged groups on the cytosolic side of the plasma membrane. Due to this property K-Ras4B can escape the trafficking through Golgi; the exact mechanism is however not clear (Cox and Der 2010).

Several studies revealed a connection between the specific modifications on Ras isoforms and their membrane localisation like in the case of H- and N-Ras where their different palmitoylation levels determine N-Ras localisation to the cis-Golgi and H-Ras distribution throughout the Golgi membranes (Lynch, Snitkin *et al.* 2015). Moreover, different lipid domain organisations were found for these two isoforms when they are bound to GDP or GTP. These lipid domains were organised in rafts only when H-Ras was bound to GDP and N-Ras to GTP (Rotblat, Prior *et al.* 2004, Eisenberg, Beckett *et al.* 2011).

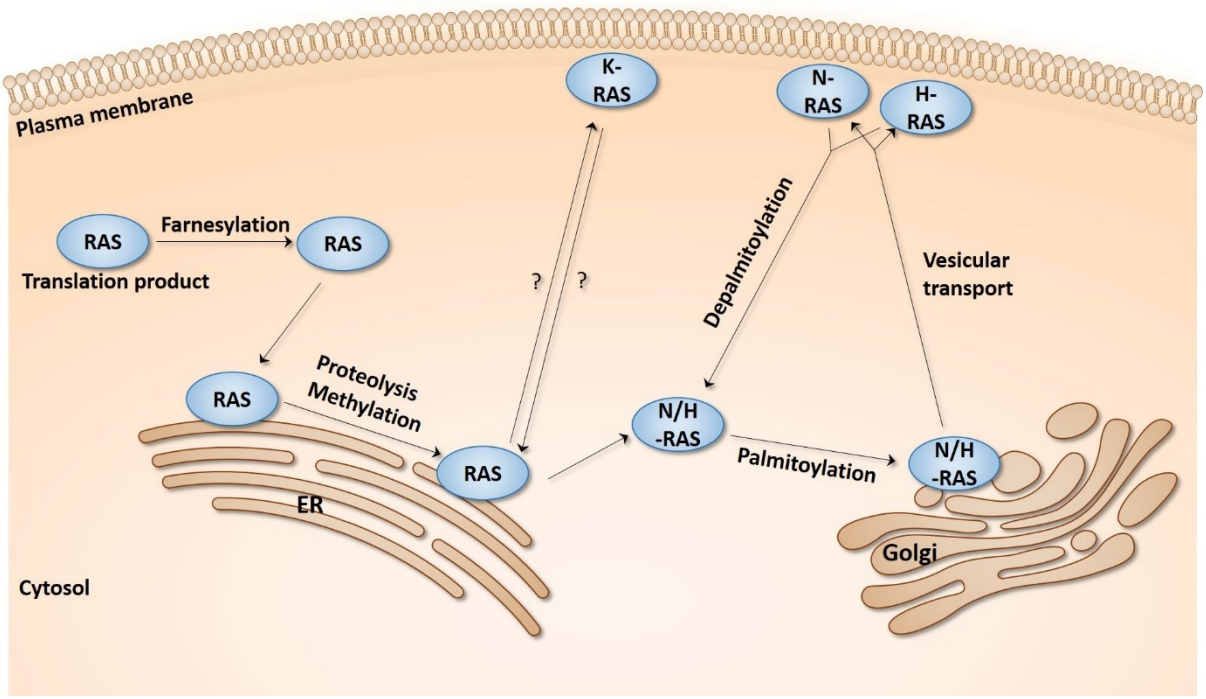

Figure 2. Post-translational modifications of Ras proteins.

Processing of Ras starts in the cytosol by farnesylation. It then translocates to the ER where it undergoes proteolysis and methylation. By means of an unknown mechanism, K-Ras traffics to the plasma membrane, whereas N/H-Ras palmitoylate in the Golgi apparatus and by means of transport vesicles attach themselves to the plasma membrane. After depalmitoylation N/H-Ras reenter the Golgi apparatus to palmitoylate again. RAS: H/K/N-Ras

There are many other modifications on the hvr of Ras proteins not related with their translocation to the plasma membrane, but mainly affect the biological activity and interaction with downstream effectors. For example, K-Ras4B is the only isoform that has a Serine phosphorylation site at position 181 responsible for its translocation to endomembrane compartments. Phosphorylation sites found in H-Ras at position Y137 and N-Ras at position Y32 altered the affinity of the Ras-binding domain of Raf protein (Ting, Johnson *et al.* 2015, Bunda, Heir *et al.* 2014, Downward 2003, Cox and Der 2010, Hobbs, Der *et al.* 2016).

Ubiquitylation of Ras isoforms, as another form of post-translational modification, was found to have different effects on Ras activity in a cell type dependant manner. In 2006 Jura *et al.* showed that ubiquitylation of H-Ras induced its internalization (Jura, Scotto-Lavino *et al.* 2006), while few years later Baker *et al.* demonstrated that ubiquitylation at K117 position

enhanced its activation (Baker, Lewis *et al.* 2013). Monoubiquitylation of K-Ras at K147 was first though to accelerate GTP binding on Ras and consequently its interaction with downstream effectors. In 2015 it was shown that the real reason behind this Ras hyperactivation was an altered and weakened response to GTPase-activating proteins.
As a final form of modification, K-Ras4B was found to be acetylated at position K104. Yang *et al.*, identified an impaired conformation of Switch II region leading to a reduced activation of the protein (Yang, Nickerson *et al.* 2012).

1.6. Guanine nucleotide exchange factors and GTPase activating proteins

As mentioned earlier, Ras proteins have an intrinsic ability to cycle between the GDP and GTP-bound states. The speed of this reaction was estimated to be about 30min, a rather slow turnover rate taking into account the kinetics of endogenous Ras activation (seconds to minutes) (Feuerstein, Goody *et al.* 1987, Trahey, Milley *et al.* 1987, Neal, Eccleston *et al.* 1988, John, Schlichting *et al.* 1989). This process of spontaneous GTP hydrolysis is accelerated by two groups of enzymes: guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) that lead to the activation or inactivation of Ras protein respectively (Wittinghofer and Waldmann 2000). The interaction between Ras protein and GEFs/GAPs initiates with the translocation of the latter from the cytosol to the plasma membrane where the G protein is localised. The plasma membrane recruitment of Son of Sevenless (SOS), one of the best characterised RasGEF, is mediated by Grb2 (adaptor growth factor receptor-bound 2) with which SOS is constitutively bound. The SH2 domain of Grb2 brings the complex to the activated receptor tyrosine kinase. On the other hand, the plextrin homology (PH) and histone homology domains of SOS support the translocation due to their affinity for negatively charged phospholipids.
Similarly, the SH2 domain of the first identified RasGAP (p120GAP) is responsible for its recruitment to the plasma membrane by binding to the phospho-tyrosine on the receptor tyrosine kinase and placing p120GAP in close proximity with RasGTP. This mechanism does not apply to all RasGAPs. Membrane localization of neurofibromin 1 (NF1), another RasGAP, is achieved after its binding with Spred1. This interaction involves the non-catalytic portion of GAP-related domain of NF1 and the N-terminal Ena/VASP Homology 1 (EVH1) domain of Spred1 (Dunzendorfer-Matt, Mercado *et al.* 2016).

Given that Ras protein shows no preference or higher affinity for GTP or GDP and also the intracellular GDP:GTP ratios ranging between 1:10 to 1:50, nucleotide exchange would occur in favour of GEF-mediated accumulation of active Ras-GTP (Trahey and McCormick 1987, Bos, Rehmann *et al.* 2007). This reaction is reversed by the intrinsic GTPase activity of Ras and accelerated by GAPs. It is noteworthy that all GEFs and GAPs share a considerably large region of the interaction site with Ras, leading to the observation that Ras interaction with more than one GEF and/or GAP cannot happen simultaneously (Antonny, Chardin *et al.* 1991, Gideon, John *et al.* 1992, Allin, Ahmadian *et al.* 2001, Hennig, Markwart *et al.* 2015).
A common feature of these two protein classes is their multi-domain structure and the high specificity to each G protein family, for instance Ras-GEFs have a CDC25 homology domain (CDC25-HD) combined with a Ras exchange motif (Uddin, Hussain *et al.* 2005), while Rho-GEFs contain a DH-PH tandem domain. In some cases few domains are shared between the GEFs and GAPs of different families, presumably to facilitate the crosstalk between various signalling pathways (Bos, Rehmann *et al.* 2007).

1.6.1. General mechanism of GEFs

Guanine nucleotide exchange factors (or GEFs) catalyse the GTP-loading of small GTP-binding proteins such as Ras. Several groups studied the interaction between G proteins and their corresponding GEF along with a nucleotide in binary complexes (RanGEF RCC1 and Ran) and demonstrated that G proteins have a high affinity for both. On the other hand, the interaction GEFs/nucleotide-bound G protein and nucleotide/GEF-bound G protein is weak. Thus, the G protein interaction with either one of them weakens the affinity for the other. Structural studies showed that GEFs function is to facilitate the nucleotide exchange by interfering with the P loop. To summarize, the nucleotide is strongly bound between the two Switch regions that interact with the P loop, phosphate group and Mg^{2+} ion. Once GEFs bind to the G protein, the Switch regions and the P loop undergo a conformational change occluding the magnesium-binding site. All the structural changes act as an inhibitor for phosphate binding and subsequently enhancing the nucleotide release (Vetter and Wittinghofer 2001, Bos, Rehmann *et al.* 2007). An additional player in this reaction is a glutamine residue in the Switch II region directed toward the phosphate groups, which enhances the release of the nucleotide.

1.6.2 General mechanism of GAPs

GTPase-activating proteins (or GAPs) expedite GTP-hydrolysis of G-proteins by enhancing their enzymatic activity. The investigation of Ras interaction with a non-hydrolysable GTP analogue yielded initial hints in understanding the mechanism of action of GTPase-activating proteins. NF1 and p120GAP are the first and best characterised RasGAPs. Structural studies demonstrated that GAP's arginine residues play an important role in the activation of Ras GTPase. More specifically Arg 1276 in NF1 and Arg 789 in p120GAP, termed arginine-fingers, attracted most of the attention as they were identified as the major contributors for the GAP-mediated catalysis and were highly conserved among GAPs (Ahmadian, Stege *et al.* 1997). Further investigations demonstrated that the orientation of the water molecule in front of the γ-phosphate group is coordinated by the interaction between RasGAP and the Glutamine61 of Ras. Subsequently the water molecules are polarised and occluded from the active site. This transition state is further stabilized by the arginine-finger of the RasGAP already situated in the phosphate-binding site where it also neutralises its negative charge. The structure of GAPs is not well conserved leading to differences on how they approach and support GTPase activity of G proteins. However the general mechanism remains the same emphasizing the fact that GAPs function is mainly in supporting and stabilizing the transition state by supplying the catalytic arginine (Vetter and Wittinghofer 2001, Bos, Rehmann *et al.* 2007).

1.7. Ras signalling pathways

Ras plays an important role in transmitting signals from the extracellular environment inside the cells and activating several pathways that result in cell growth, proliferation, survival, differentiation, migration and apoptosis (Fig.3). By virtue of its position at the plasma membrane it is amenable to activation by epidermal growth factor receptors (EGFR), T cell receptors or G protein coupled receptors. One of the best characterized signalling pathways most affected by Ras activity is the mitogen-activated protein kinase cascade (MAPK). The activation of this pathway starts when a ligand binds to the respective receptor tyrosine kinase, for example EGF (epidermal growth factor) binding on EGFR (Merchant, Voskresensky *et al.* 2008). EGF binding induces dimerization and phosphorylation of the receptor that in

turn interacts and binds to the SH2 domain of the adaptor protein Grb2 (Reuter, Morgan *et al.* 2000). The latter is associated with the RasGEF SOS through its SH3 (Src homology 3) domain, after being recruited to the plasma membrane. Ras, by default localized to the plasma membrane and in proximity with SOS, is then activated by exchanging GDP with GTP (Zenonos and Kyprianou 2013). Active Ras has the ability to recruit and bind Raf protein. As for Ras, Raf localization in proximity to the plasma membrane is essential for its proper function. The interaction between Ras and Raf leads to the release of 14-3-3 protein that is normally bound and therefore inhibits Raf. Once active, Raf forms heterodimers that induce translocation of the KSR1 (kinase suppressor of Ras) enzyme to the plasma membrane (Nandan and Yang 2011). KSR is a scaffold protein that is stably bound to MEK and its translocation brings MEK close to its activator – Raf (Morrison 2001). Consequently, MEK is phosphorylated and is able to further activate Erk. Phosphorylated Erk can act in the cytosol as well as in the nucleus. Most of the research has been focused on understanding how Erk can regulate transcription factors. Once inside the nucleus, Erk can regulate the expression of FOS transcription factor and phosphorylate c-Jun. Both of them bind to the AP1 transcription factor that leads to the expression of genes involved in proliferation like CyclinD1 (Downward 2003).

PI3K pathway can also be stimulated by active Ras. Normally, PI3Ks are composed of a regulatory and catalytic subunit, generally referred to as p85 and p110 respectively, which are activated by receptor tyrosine kinase by directly binding a phosphorylated residue on the receptor or an adaptor (Katso, Okkenhaug *et al.* 2001, Engelman, Luo *et al.* 2006, Yuan and Cantley 2008). This interaction is mediated by p85, whereas p110 converts phosphatidylinositol 4,5-bisphosphate (PIP_2) into phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Carpenter, Auger *et al.* 1993, Zhao and Vogt 2008). Several studies have shown that active Ras is able to directly bind catalytic subunit p110, activating in this way the whole pathway (Shaw and Cantley 2006). Production of PIP_3 induces the recruitment of two serine/threonine kinases, PDK1 and Akt. Phosphorylation of Akt at position Threonine308 by PDK1 (Alessi, James *et al.* 1997, Currie, Walker *et al.* 1999, Majumder and Sellers 2005) promotes survival through the following mechanisms: 1. inhibiting pro-apoptotic Bcl-2 family members (Cantley 2002, Engelman, Luo *et al.* 2006); 2. enhancing transcription of anti-apoptotic and pro-survival genes by preventing the negative regulation of NF-kB; 3. inhibiting p53-mediate apoptosis by Mdm2 phosphorylation (Duronio 2008); 4. blocking FOXO

(forkhead box transcription factor) activity responsible for the transcription of proteins that promote cell death; and 5. phosphorylation of TSC2 (tuberous sclerosis complex 2) that leads to the subsequent activation of Rheb-GTP and mTORC1 complex (mammalian target of rapamycin complex 1) to increase protein synthesis (Engelman, Luo *et al.* 2006). In parallel, mTORC2 (mammalian target of rapamycin complex 2) sustains Akt activity by further phosphorylating the protein at position Serine 473 (Hresko and Mueckler 2005, Sarbassov, Guertin *et al.* 2005, Sarbassov, Ali *et al.* 2006). A negative feedback is initiated from one of the targets of mTORC1, S6 kinase, to terminate the PI3K signalling at the level of the adaptor protein on the cytoplasmic part of the RTKs (Harrington, Findlay *et al.* 2004, O'Reilly, Rojo *et al.* 2006, Carracedo and Pandolfi 2008, Courtney, Corcoran *et al.* 2010).

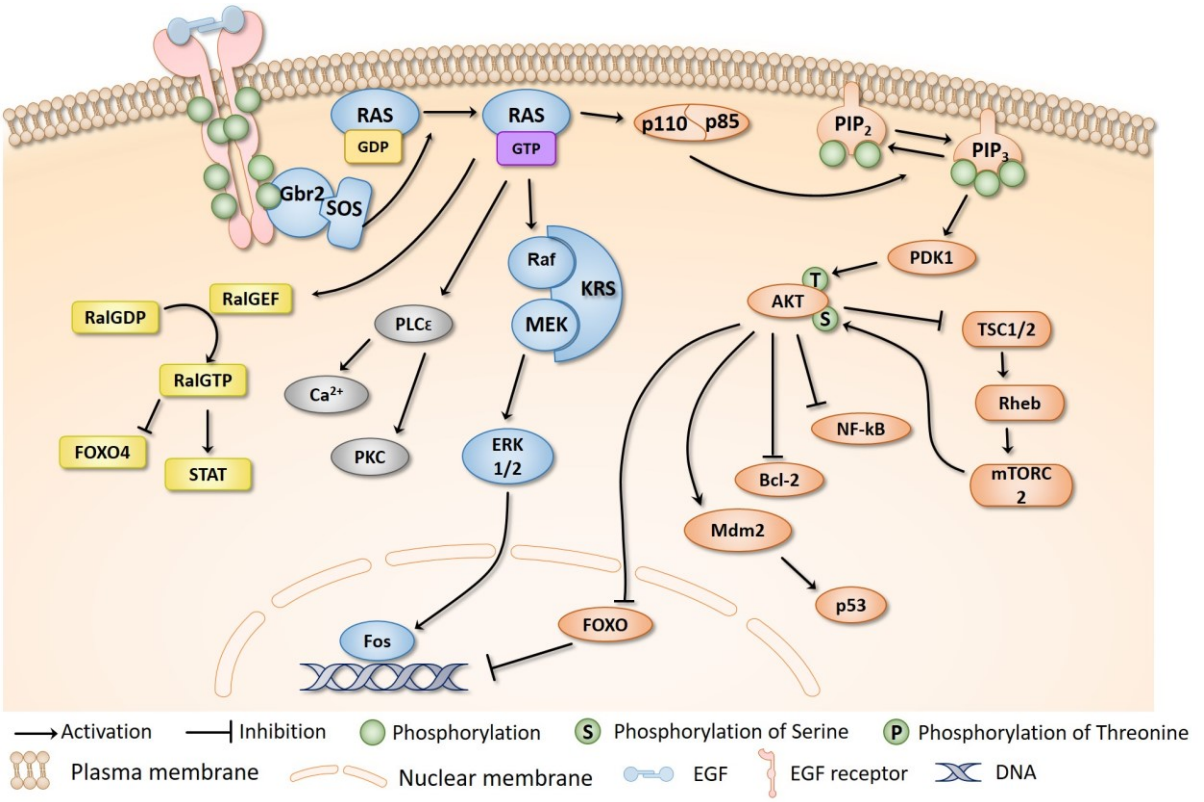

Figure 3. Scheme of Ras signalling pathways.

EGFR activation by its ligand leads to GTP loading of Ras mediated by Grb2/SOS proteins. The 2 most important downstream pathways, MAPkinase and PI3K-AKT are activated by GTP-bound Ras and induce or inhibit gene transcription. Other Ras mediated pathways are the Ral and PLCε cascades. For further details refer to section 1.7.

A third signalling network altered by Ras activity is the RalGEF-Ral effector pathway (Albright, Giddings *et al.* 1993). In this case, in contrast to the previously described crosstalks, the

downstream effector of Ras is a member of the GEF family, RalGDS (Ral guanine nucleotide dissociation stimulator). Ral is another small GTP-binding protein that transitions between GTP-bound active and GDP-bound inactive state. Identification of RalGDS as an effector of Ras was confirmed by the introduction into the cell (COS7) of a mutant Ras (RasV12G37) that stimulated RalGEF activity (Urano, Emkey *et al.* 1996, Murai, Ikeda *et al.* 1997, Bos 1997). Moreover, RasV12G37 displayed a high selectivity for RalGEF and did not interact with neither Raf nor PI3K. Even though the two Ral proteins, A and B, share a 100% sequence identity in the effector binding site, they have different functions within the cell especially due to their distinct localisation. RalA is mostly found localized close to the plasma membrane and endosomes, while RalB is primarily associated with endosomes (Shipitsin and Feig 2004). Nonetheless, depending on its activation state or phosphorylation Ral localization can vary (Cascone, Selimoglu *et al.* 2008, Lim, Brady *et al.* 2010). The best characterized Ral function is its involvement in regulating exocytosis. Further studies showed a direct activation of phospholipase C delta 1 and D1 (Luo, Liu *et al.* 1997, Sidhu, Clough *et al.* 2005). The latter increases production of lipid secondary messengers. Another effector of RalA, even though less characterized, is filamin involved in actin reorganization. Expression of several genes is regulated by this signalling pathway and mediated by myriad transcription factors like c-Jun activation, FOXO4, STAT3, c-Fos and NF-kB (Neel, Martin *et al.* 2011).

Phosphoinositol-specific phospholipase C (PLC) is a signalling enzyme that induces production of PIP3 which in turn increases intracellular Ca^{2+} and DAG (diacylglycerol). The latter is able to activate several isoforms of protein kinase C (PKC). Up to date, four different classes of PLC are identified: β, γ, δ and ε. Kelley *et al.* demonstrated that PLCε was directly regulated by Ras through its C-terminal Ras-binding domain in a GTP-dependent manner (Kelley, Reks *et al.* 2001).

1.8. Ras mutations in cancer

The first oncogenes discovered in human cancer were Ras genes. 30 years of studies classified Ras genes as one of the most mutated driver genes in cancer. Although the first Ras isoform to be identified as an oncogene was H–Ras, further investigation showed that H-Ras had in fact the lowest frequency, whereas K-Ras had the highest (Stephen, Esposito *et al.* 2014). Despite the high degree of sequence homology between the four isoforms of Ras proteins,

over the years the idea of functional specificity for each isoform always became clearer. Many studies demonstrated that different types of tumours or developmental diseases showed a preference for one of the isoforms leading to a cell-type dependent transformation potential. Differences in post-translational modifications, subcellular localization and crosstalk with distinct transcriptional signalling pathways helped understanding that Ras isoforms acted separately and without overlapping with each other's functions (Castellano and Santos 2011). 27% of human cancer have missense gain-of-function in Ras genes at one of the hotspots G12, G13 and Q61. They count for 98% of all mutations found so far and result in a hyperactive Ras phenotype. In addition, dysregulation of GDP-GTP binding as well as loss of GAPs and constant GEFs activation increases Ras involvement in cancer development. As mentioned earlier, the mutation frequency of Ras isoforms is different with K-Ras being mutated in 85% of human cancers, N-Ras in 11% and H-Ras in only 4%. Numerous investigations showed evidence of cancer-specific mutation profiles where K-Ras was mutated in pancreatic ductal, lung and colorectal carcinomas; N-Ras mutations are found primarily in cutaneous melanoma while H-Ras in head and neck squamous cell carcinoma (Table 1.) (Hobbs, Der *et al.* 2016).

Table 1. Ras mutations in cancer (Hobbs, Der *et al.* 2016)

Cancer	**Isoform**	**Percentage**
Pancreatic ductal adenocarcinoma	K	98
Colorectal adenocarcinoma	K	52
Multiple myeloma	K, N	43
Lung adenocarcinoma	K	32
Skin cutaneous melanoma	N	29
Uterine carpus endometrioidcarcinoma	K	25

Uterine carcinosarcoma	K	14
Thyroid carcinoma	N > H	13
Acute myeloid leukemia	N > K > H	11
Bladder urothelial carcinoma	H, K > N	11
Gastric adenocarcinoma	K	10
Cervical adenocarcinoma	K	8
Head and neck squamous cell carcinoma	H	6

1.9. Ras in cell cycle control

1.9.1. Cell cycle control machinery

Cell cycle is the essential mechanism of reproduction in living organisms. The major events of the eukaryotic cell cycle performed with high accuracy are chromosome duplication or DNA synthesis (S phase) and physical cell division or mitosis (M phase). One single cell cycle in a typical proliferating eukaryotic cell lasts an average of 24 hours, of which only 1 hour is occupied by mitosis and 10 to 12 hours by S phase. The remaining time is divided between two preparatory gap phases (G1 and G2) that precede S and M phase, respectively. During this time, the cell grows in size, duplicates intracellular organelles and proteins and monitors the intra-/extracellular environment to ensure that all conditions are in favour of DNA synthesis or mitosis. G1 phase, usually longer than G2, is highly dependent, but not only, on extracellular stimuli. For example is case of limited growth factors availability, cells can delay G1 progression or even decide to exit the cell cycle and enter a resting state known as G0 (quiescence) (Berridge 2014). The orderly progression through G1-S-G2-M is strictly controlled by a complex machinery whose main components are cyclins and cyclin-dependent kinases (CDKs). The cell cycle machinery is responsible for the transition through 3 crucial moments of the cell cycle: G1/S, G2 and spindle checkpoint. Progression through G1 and S

phase transition is supported and controlled by two complexes of cyclin-CDKs (cyclinD-CDK4/6 and cyclinE-CDK2) in cooperation with the tumour suppressor pRB (retinoblastoma protein) (Weinberg 1995). Active pRB, hypophosphorylated state, is always bound to E2F transcription factor to repress its activity. pRB-E2F complexes are stable in quiescent cells and those exiting from mitosis. Mitogen–induced activation of cyclinD-CDK4/6 and cyclinE-CDK2 complexes cause hyperphosphorylation of pRB, thus releasing E2F factors that promote expression of genes required for S phase entry (Dyson 1998, Coleman, Marshall *et al.* 2004). Once the G1/S checkpoint is crossed, the cell is committed to complete DNA synthesis and progress to the second gap phase. G2 phase and G2/M transition is further controlled by a CyclinB-CDK1 complex (Duronio and Xiong 2013). The cell cycle machinery has several layers of complexity and is highly orchestrated to execute progress through each phase of the cell cycle. A rather simplified view of its elaborate regulation is presented in figure 4.

Despite the commitment to complete the cell cycle, in the event of any untoward anomaly, cells can activate both local control (for e.g., at the site of DNA replication factories) and global control via the next closest checkpoint to allow time for repair and restoration of the damage, failing which apoptosis is the chosen fate of the cell (Malumbres and Carnero 2003). As fail-safe as these regulatory measures are, occasional errors that escape checkpoint surveillance may lead to uncontrolled cell proliferation and cancer. One of the most common characteristics in cancer development is dysregulation in G1 control (Sherr 1996), often connected to alterations of the tumour suppressor pRB like mutations and deletions that lead to loss of function of the protein. Although the dramatic dysregulation in cell cycle is a characteristic of every cancer, it is often a consequence and not sufficient to drive tumorigenesis in the first place.

1.9.2. Ras-dependent cell cycle progression

In 1985, Ras was identified for the first time as a key protein in cell cycle regulation. Ras function in cell cycle entry and progression was investigated in continuously proliferating cells and those emerging from quiescence. Neutralizing Ras activity via antibody microinjections or expression of inhibitory mutants stopped entry in S phase upon serum stimulation in cycling cells (Mulcahy, Smith *et al.* 1985, Stacey, Feig *et al.* 1991). Quiescent cells on the other

hand, were induced to proliferate only in the presence of oncogenic Ras and lack of growth factors (Feramisco, Gross *et al.* 1984, Mulcahy, Smith *et al.* 1985).

The G1 to S phase transition was established to be Ras-dependent due to its ability to release E2F transcription factor from the inhibitory effect of pRB by hyperphosphorylating the latter (Mittnacht, Paterson *et al.* 1997, Peeper, Upton *et al.* 1997, Coleman, Marshall *et al.* 2004). However, pRB activity is also controlled by the PI3K pathway which upon inhibition can induce G1 arrest, unless pRB function is disrupted. Conversely, Ras activity but not Erk signalling, is still required in pRB-deficient cells to depart from quiescence and start proliferating (D'Abaco, Hooper *et al.* 2002). These findings suggested that under specific conditions Ras might have a different effector pathway. A second key player in this mechanism is cyclinD1 that in assembly with CDK4 or CDK6 directly hyperphosphorylate pRB. Many studies describe cyclinD1 expression being dependent on Ras signalling (Coleman, Marshall *et al.* 2004). They have different Ras requirements depending on the position of cells in the cycle. More specifically, cyclinD1 expression is dependent on Ras activity in resting cells re-entering the cycle (Filmus, Robles *et al.* 1994), while for actively proliferating cells Ras-induced cyclinD1 occurs in the preceding G2 phase (Fig. 4) (Hitomi and Stacey 1999b). Ras protein exerts its effect on cyclinD1 transcription through two different signalling pathways: MAPK and PI3K pathways (Gille and Downward 1999). Erk-MAPK signalling facilitates cyclinD1 transcription (Suzuki, J *et al.* 2002), whereas PI3K the translation of cyclinD1 mRNA (Muise-Helmericks, Grimes *et al.* 1998). Yu *et al.* demonstrated that cyclinD1 is a prerequisite for Ras signalling to induce proliferation. They showed that cyclinD1-deficient mice did not develop breast cancer in presence of oncogenic Ras (Yu, Geng *et al.* 2001).

Ras-mediated proliferation signals in G1 phase also alter p27 activity toward the end of G1 phase by downregulating its overall expression and inducing binding with cyclinD1-CDK4 complex. As a result, CyclinE-CDK2 disassociates from p27 by becoming active and inducing S phase entry (Aktas, Cai *et al.* 1997, Takuwa and Takuwa 1997, Weber, Hu *et al.* 1997). Somewhat contrary to this, p21 is observed to be marginally upregulated upon growth factor stimulation (Liu, Martindale *et al.* 1996, Bottazzi, Zhu *et al.* 1999). This effect might be explained with the role of p21 in stabilising cyclinD1-CDK4 complex (Cheng, Olivier *et al.* 1999).

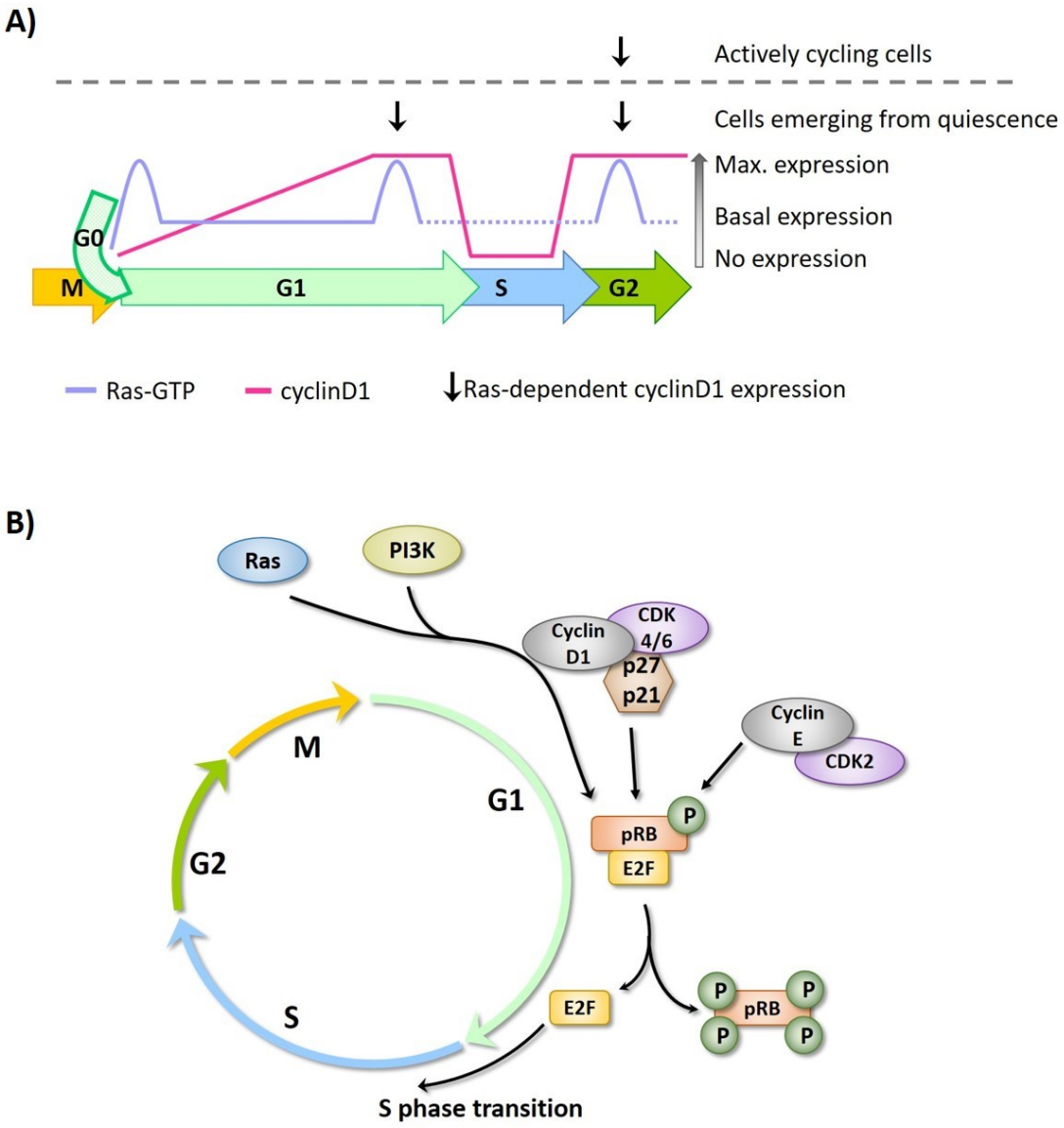

Figure 4. Ras activity during the cell cycle progression.

A) Ras activity reaches two genuine peaks in the early and mid/late G1 phase, respectively. Late G1 phase is also characterized by a high CyclinD1 expression. For cells emerging from quiescence, Ras is required to induce cyclinD1 expression in G1 and G2 phase. In contrast, for actively cycling cells cyclinD1 expression is depend on Ras only during the preceding G2 phase. B) G1 to S transition is highly dependent on the hyperphosphorylation of the retinoblastoma protein (pRB) that releases the transcription factor E2F. Hyperphosphorylation of pRB is catalysed from the two cyclin-CDK complexes: cyclinD1-CDK2/4 and cyclinE-CDK2. Ras and PI3K signalling are two major pathways involved in the in controlling pRB cycling between its hypo- and hyper-phosphorylated forms, through cyclinD1.

Despite the generally accepted mechanism, that Ras inactivates pRB by inducing a subsequent cyclinD1 expression and cyclinE activation, development of the mouse embryonic fibroblasts

(MEFs) carrying genetic ablation of the three Ras genes described a different expression pattern for the regulators of the cell cycle. Drosten *et al*. demonstrated that in the total absence of Ras protein (Rasless), MEF cells underwent significant changes in their morphology similar to senescent cells but did not express any specific marker (Drosten, Dhawahir *et al.* 2010). Furthermore, Rasless cells could be held in culture for several weeks without activating apoptotic pathways. The special features of these Rasless MEFs were the normal expression levels of cyclinD1, its CDK associated complexes and cyclinE-CDK2. Nonetheless, phosphorylation of pRB did not occur, blocking the cells in a non-proliferating state. Only external downregulation of pRB could restore their proliferative capacity. These results indicated an important role of Ras proteins on controlling cyclin-CDK enzymatic activity mediated possibly through CDK inhibitors. shRNA (small hairpin RNA) library screen in Rasless cells highlighted the p53-p21Cip1 tumour suppressor axis as a new effector of Ras signalling (Drosten, Sum *et al.* 2014). Loss of either p53 or p21Cip1 expression induced proliferation in these cells presumably through a retroactive loop that sustained an active MAPK cascade independent from Ras (Drosten and Barbacid 2016).

It is now well accepted that quiescent cells require growth factor stimulation not only to enter G1 phase, but also later until they cross the late G1 restriction point when cell become committed to progress through S phase (Pardee 1974). Injection of antibodies against Ras and cyclinD1 in NIH3T3 cells released from quiescence inhibited S phase entry at the same time. Interestingly, entry in S phase of cycling cells was blocked only when injection of anti-Ras antibodies was performed in mitosis preceding the current G1 phase. In contrast, injection of anti-cyclinD1 antibodies in G1 phase were able to significantly inhibit the next mitosis. These observations suggested distinct requirements of Ras activity and cyclinD1 throughout the cell cycle in actively proliferating NIH3T3 cells, where Ras activity is necessary in the preceding G2 phase and cyclinD1 during G1 (Hitomi and Stacey 1999b). Nonetheless, Ras was found to be constantly active throughout the cell cycle, while cyclinD1 in G1, G2 and M phase. Expression of oncogenic Ras as well as anti-Ras antibodies revealed that cyclinD1 was induced by Ras only in G2 phase and remained stable during the next G1 phase even in the absence of Ras activity (Hitomi and Stacey 1999a, Sa, Hitomi *et al.* 2002) .

1.10. Strategies to target Ras protein

Direct and indirect approaches have been developed over the years to target oncogenic Ras (Figure 5). First trials were oriented in directly blocking Ras expression using gene silencing techniques. The use of RNA interference (RNAi) and antisense oligonucleotides (ODNs) revealed a higher specificity of RNAi for its target and significant growth inhibition for several types of cancer in cell lines and animal models. Nevertheless, RNAi-based therapies are very challenging because exogenous RNA can be recognized and destroyed by the host's immune system. Additionally, it was shown that silencing of Ras was not enough to stop cancer development and eventually kill it.

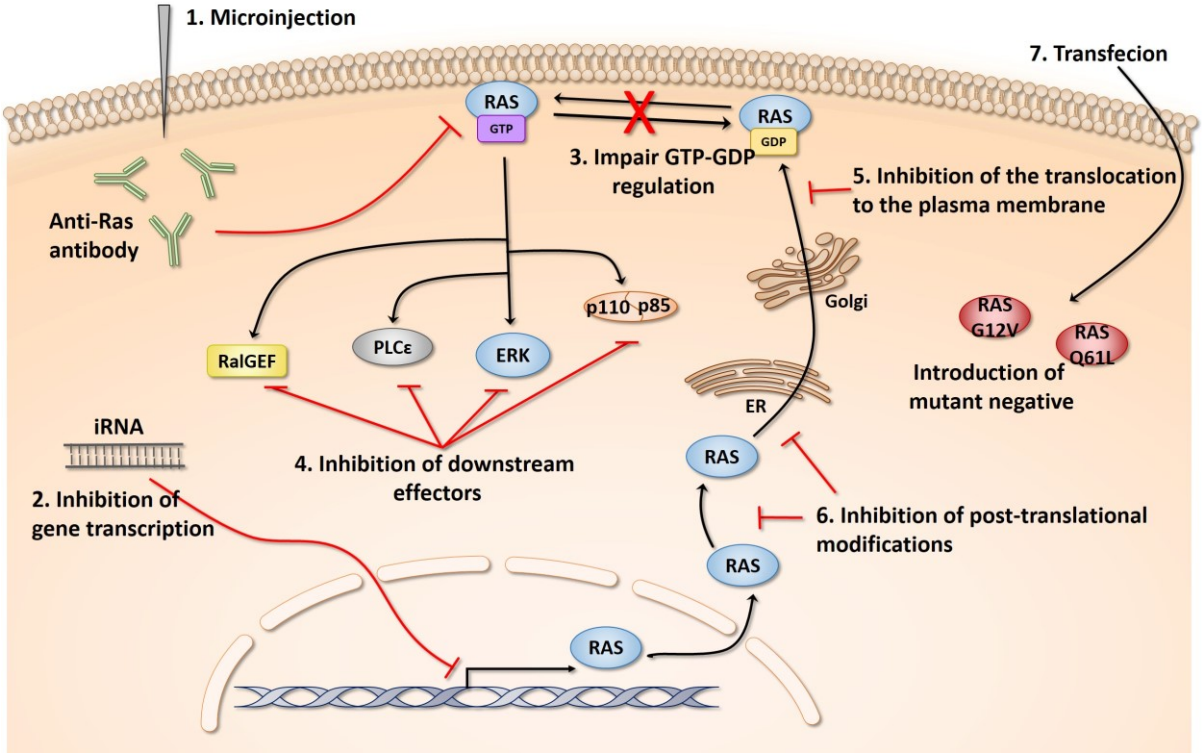

Figure 5. Schematic representation of the techniques used to target Ras protein.

1: Microinjection of specific anti-Ras antibodies that bind and inhibit Ras activity. 2: Gene silencing methods to block Ras expression. 3: Introduction of external molecules to impair GTP-GDP regulation of Ras and terminate its signalling. 4: Utilization of various inhibitors against Ras downstream effectors. 5, 6: Altering post-translational modifications to avoid Ras trafficking and attachment to the plasma membrane. 7: Transient transfection of dominant negative forms of Ras.

An alternative way to inhibit Ras was to prevent post-translational modifications that facilitate Ras translocation and association to the plasma membrane. Two groups of enzymes were targeted: FTIs and GGTIs. Failure for this strategy was due to the lack of Ras specificity

and clinical activity. Despite many unsuccessful trials, several groups tried developing small molecules that could permanently bind to inactive Ras, impairing GTP-GDP regulation and subsequently terminate signal transduction of Ras-Raf-MAPK pathway. In addition, interfering with Ras interaction with its GEFs was seen as a blocking option. One of the major strategies used to interfere with Ras signalling was targeting its downstream effectors. Some of the investigational drugs used to target Ras effectors are listed in table 2 (adapted from Takashima *et al*. 2013). The discovery and development of new drugs that target Ras-effectors is the most promising therapy so far, even though cancer cells gained resistance to some of the drugs (Takashima and Faller 2013).

Table 2. Some of the drugs used to target Ras effectors

Target protein	**Name of the drug**
MEK1/2	Trametinib Selumentinib Pimasertinib Refamentinib TAK-733 AS703988
Mutant B-Raf	Dabrafenib Regorafenib LGX818 RO5212054 ARQ736
Class I PI3K and mTORC1/2	PF-04691502 PKI-587 PF-05212384 XL765 GSK2126458 DS-7423

mTORC1/2	CC-223 ME-344 AZD2014 OSI-027
AKT1/2/3	GSK2110183 GDC-0068 AZD5363 ARQ092 GSK2141795

Another tool in cancer gene therapy was the use of dominant active and negative Ras mutants. The most frequent dominant active mutants are RasG12V and RasQ61L, while RasS17N is the most popular dominant negative mutant. Ras mutants mainly served to shed light on its role in the signalling transduction pathways and identification of downstream effectors. As mentioned in the previous section, Ras was identified as a crucial player in regulation cell proliferation. First investigations were performed by Stacey's group and made use of anti-Ras antibodies microinjected into single cells.

A general limitation of the strategies described is that they are highly time-dependent and from a technical point of view the time required to perform any experiment is on the range of 12-24 hours, sometimes even 48 hours.

1.11. Protein translocation and dimerization importance in signal transduction

Protein-protein interaction is the basis of all signal transduction networks. Depending on the extracellular stimuli and type of the cell, the biological outcome of these interactions varies. Regardless of this, protein interaction can be stable or dynamic like phosphorylation and nucleotide exchange. Two or more proteins can be engaged at the same time in every step of the signal transduction. One of the interactions during the transmission of extracellular signals to the nucleus is protein dimerization. Optimal proximity and correct orientation are two important factors that can accelerate the reaction. Sometimes this is not enough because the viscosity of the intracellular environment affects the speed of the dimerization process, usually by slowing it down. Therefore, another stimulus or mechanism that induces the

dimerization effectively helps the interaction. The most frequent example is the EGF cell surface receptors. EGFRs consist of three domains: extracellular, transmembrane and intracellular domain. Binding of the epidermal growth factor on the extracellular domain induces the dimerization of two receptors followed by their activation and further transmission of the signal (Klemm, Schreiber *et al.* 1998).

Putyrski and Schultz showed that a crucial event during the dimerization is the translocation of the proteins (Putyrski and Schultz 2012). One common example of the importance of protein translocation in signal transduction is the Akt and PDK1 interaction, which after being recruited to the plasma membrane exert their function by phosphorylating each other and forwarding the signal to the next step of the cascade (Ding, Liang *et al.* 2010). The translocation to the plasma membrane is facilitated by the second messenger PtdIns(3,4,5)P_3 (phosphatidylinositol 3,4,5-trisphosphate), that both of them recognize. Akt and PDK1 kept phosphorylating each other even when they were brought together in an artificial way in the cytosol. In contrast to other techniques like RNAi or transfection when the result of the manipulation is visible after 12 to 72 hours (Ouyang and Chen 2010, Raina and Crews 2010), the translocation took place within seconds affecting the outcome of the pathway. These observations led to the development of new and more sophisticated methods that induce intracellular translocation as a faster way to alter signal transduction.

1.12. Rapamycin/Rapalog induced translocation

Understanding the mechanism of action of immunosuppressive drugs like rapamycin gave the first hint for the development of induced dimerization systems. Rapamycin is a potent, selective, allosteric inhibitor of the TORC1 complex by binding both components of the complex: protein kinase TOR and FKBP12 (FK506 binding protein of 12 kDa). The latter is the first to interact with rapamycin following which the rapamycin-FKBP12 complex can bind to TOR (Choi, Chen *et al.* 1996, Liang, Choi *et al.* 1999).

Based on this mechanism, FKBP12 and FRB (FKBP and rapamycin binding site of TOR) were fused with two other proteins to induce rapamycin-dependent gene expression in cells (Rivera, Clackson *et al.* 1996). For many years, the rapamycin-inducible FKBP-FRB interaction was used to specifically manipulate protein activity localised at the plasma membrane like G

proteins, enzymes involved in metabolism, receptor tyrosine kinases, protein kinases and adaptor proteins.

The effects of rapamycin application in cultured cells were overlooked only in case of short time experiments. Otherwise, non-toxic rapalogs (synthetic analogues of rapamycin) administration was recommended to avoid negative effects of rapamycin on cell growth, proliferation and apoptosis (Wullschleger, Loewith *et al.* 2006, Zoncu, Efeyan *et al.* 2011). The rapalogs design was based on the "bump-and-hole" scheme (Clackson 1998, Clackson 2008). The FRB-binding site of rapamycin (the "bump") was modified in order to lose its affinity to recognise and bind the wild type FRB. On the other hand, the rapamycin-binding pocket (the "hole") was enlarged to fit the rapalog as well as to impair interaction with rapamycin itself. Among the several rapalogs produced, the most used is AP21967. Despite all the advantages that this new technique introduced, there are some limitations. Among them is the high affinity between rapamycin or rapalogs and their target proteins that makes the reversibility of the heterodimerization system a difficult task to accomplish. Up to date, only few papers have reported a dissociation of the fusion proteins in the order of hours (Karpova, Tervo *et al.* 2005). It is worth mentioning, that there are no readymade fusion proteins meaning that every lab must clone its own system which is fairly challenging. Moreover, both fusion proteins should be stably introduced into the desired cell line and further checked for their correct localization and proper functionality (Putyrski and Schultz 2012).

1.13. Use of the dimerization system to study small GTPases

The induced heterodimerization system has been very useful in investigating the specific role of small GTPases due to its rapid perturbation on the functionality of its target protein (Inoue, Heo *et al.* 2005). Many groups focused their work on the activation mechanism and function of several members of the Rho family such as Rac and Cdc42. Castellano *et al.* studied Rac1 role in lamellipodia formation and membrane raffling (Castellano and Chavrier 2000, Castellano, Montcourrier *et al.* 2000). For this purpose, they used a rapamycin-induced translocation of FRB-bound Rho GTPase (Rac1) towards the FKBP-conjugated transmembrane receptor and were able to demonstrate that Rac signalling was important in remodelling the plasma membrane, thus allowing particle internalization. Since, their translocation strategy was not sufficient to prove their point, other techniques like antibody-coated beads were

used to support the heterodimerization system. Few years later, Inoue *et al.* developed an improved system able to directly target Rho GTPases in a faster way (Inoue, Heo *et al.* 2005). The key to their success was designing several fusion constructs with different orientations of the proteins and numbers of FKBP domains. In addition, they switched the FKBP and FRB domains between the two fusion proteins and used the rapamycin analog, AP21967 (iRap). By testing all pair's combinations for the iRap-induced lamellipodia extension, the best results were obtained by the heterodimerization of the FRB-coupled membrane-bound fusion protein with the FKBP- bound Rac1 fusion construct.

Komatsu *et al.* made further use of this system to activate Ras at distinct intracellular locations. Both fusion proteins were modified to bind and/or act at specific cellular compartments: plasma membrane, mitochondria, endoplasmic reticulum and lysosome. Addition of the chemical iRap induced dimerization of the fusion proteins in all organelles without differences (Komatsu, Kukelyansky *et al.* 2010).

1.14. Inducible system to switch on and off Ras

Based on the prototypic heterodimerization kit of Ariad/Clontech technologies (Inoue, Heo *et al.* 2005), our lab developed an inducible FKBP-FRB system capable of altering Ras activity by turning it off. The FKBP-coupled fusion protein is stably associated to the plasma membrane through the hvr-CAAX motif (hypervariable region) and from now on will be referred to as the '*anchor unit*'. The FRB-coupled fusion protein included a RasGAP (NF1, Neurofibromin1) that catalyses GTP expenditure to inhibit Ras function, when positioned in proximity to GTP-loaded Ras. In the absence of a specific stimulus, this fusion protein is deliberately designed to be diffusely expressed in the cytosol and will be referred to as the '*effector unit*'. A simple scheme illustrates the principle of RasOFF heterodimerization system in figure 6.

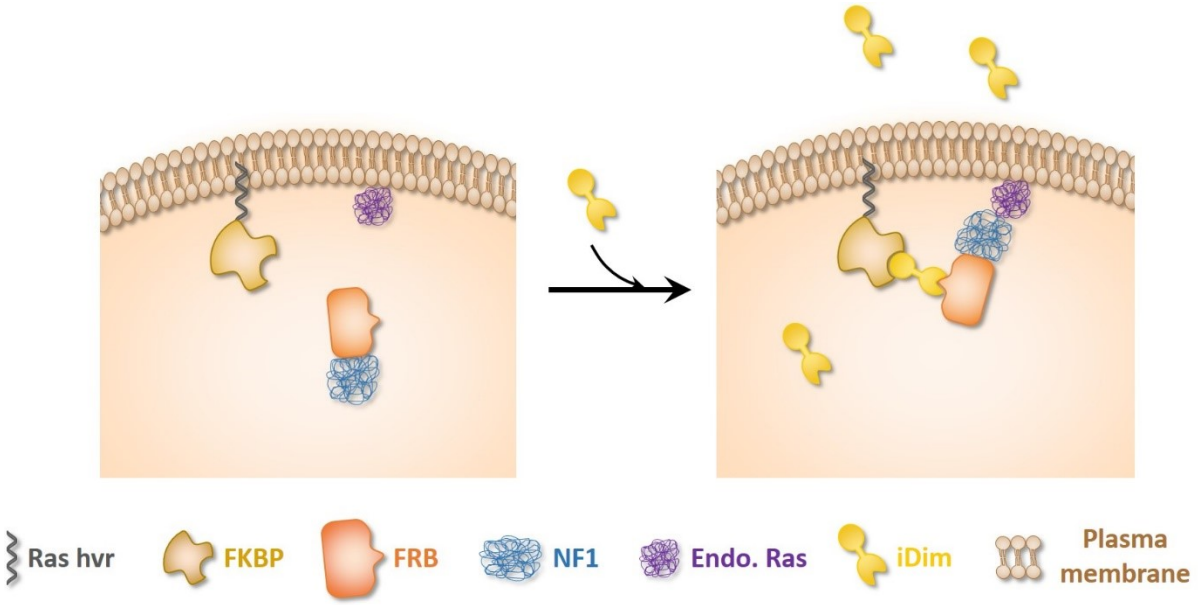

Figure 6. Principle of the induced RasOFF heterodimerization system.

FKBP-coupled anchor unit is bound to the plasma membrane through the hvr of K-Ras, while the FRB-coupled effector unit is spread throughout the cytosol. Upon heterodimerizer (iDim) addition, the effector unit translocates toward the membrane-bound unit and is able to inhibit the activity of endogenous Ras protein.

The chemical dimerizer used in our case is the commercially available rapamycin analogue, AP21967 (iDim). Administration of the latter induces the translocation of the effector unit toward the plasma membrane bringing NF1 in close proximity with endogenous Ras. Consequently, Ras should be turned off.

This heterodimerization system targets Ras in a rapid and less invasive way for the cell and provides a new, more elegant technique to investigate the function of Ras at particular stages of the cell cycle.

2. Aim of the work

Ras is a monomeric membrane-associated GTP-binding protein that fluctuates between two distinct conformational structures defining its state as active (GTP-loaded) or inactive (GDP-loaded). Sustained in its active state, Ras becomes oncogenic, inducing uncontrolled cell proliferation and cancer development. In fact, Ras mutations are among the most common, found in nearly 30% of human cancers, yet the precise nature and magnitude of Ras signalling in the temporal control of cell proliferation is only partly understood.

Seminal reports have linked Ras activity with cyclinD1 expression to enable cell cycle progression depending on the proliferative context of cells. Primarily due to the lack of appropriate experimental approaches, very little is known about the involvement of Ras signalling at precise times during particular cell cycle phases that determines the fate of proliferating cells.

In this thesis work, we aimed to gain insight into the importance of Ras signalling with respect to progression through different cell cycle stages using a unique heterodimerization system allowing rapid inactivation of Ras. The major experimental aims were as follows:

1. Validating the utility and extent of application of the heterodimerization system in studying dependent control of cell proliferation and cell cycle progression.
2. Establishing a suitable cell model amenable to efficient synchronisation, while also stably expressing the two-unit heterodimerization system for Ras inactivation.
3. Functional dissection of the role and requirement of Ras signalling through the cell cycle by abrogating its activity at specific phases.
4. Elucidating effector pathways downstream of Ras that exert its function in cell cycle control.

3. Materials and methods

3.1. Materials

Table 3. Cell lysis and flow cytometry buffers

Cell lysis buffers	**Composition**
Ras activity lysis buffer	50 mM HEPES pH 7.5 150 mM NaCl 5 mM $MgCl_2$ 1 mM EGTA 1% NP-40 Freshly added: 100µM GDP 2mM DTT 25µg/ml purified GST-Raf1-RBD
RIPA lysis buffer	50mM TRIS-HCl pH 8.0 150mM NaCl 5mM $MgCl_2$ 1% Nonidet P-40 0.5% Deoxycholate 0.1% SDS
Protease and phosphatase inhibitors	freshly supplemented from frozen stocks Protease inhibitors 42 mM Pefabloc 2 µM Leupeptin

	100 µM PMSF 1.5 µM Pepstatin A phosphatases inhibitors 100 µM Sodium Vanadate 3.4 nM Microcystin 1 µM β-Glycerophosphate
Reaction buffer for homemade EdU-ClickiT cell cycle analysis	50mM Tris HCl pH 7.6 150mM NaCl

Table 4. SDS-PAGE buffers

SDS-PAGE buffers	**Composition**
10 x electrophoresis running buffer	250 mM Tris 2 M Glycin 35 mM SDS
2 x Laemmli buffer	20% Glycerol 4% SDS 10% 2-Mercaptoethanol 0.02% Bromophenol blue 124 mM Tris pH 6.8
5 x Laemmli buffer	33% Glycerol 5% SDS 25% 2-Mercaptoethanol 0.02% Bromophenol blue 85 mM Tris pH 6.8
Separating gel buffer	2 M Tris pH 8.8
Stacking gel buffer	0.5 M Tris

	pH 6.8
Coomassie staining buffer	0.5% Coomassie Brilliant Blue R-250 in absolute ethanol mix 1:1 with 20% acetic acid
Destaining buffer	10% Acetic acid 25% Ethanol

Table 5. **Western blotting buffers**

Western blotting buffers	**Composition**
Transfer buffer	48 mM Tris 39 mM Glycin 0.037% SDS 15% Methanol pH 10
10 x TBS-Tween	100 mM Tris 1 M NaCl 1% Tween 20 pH 7.6
Stripping buffer	100 mM 2-Mercaptoethanol 62.5 mM Tris 2% SDS pH 6.7
Antibody solutions:	
Primary antibody solution	1%BSA in 1xTBS-Tween with 0.02% NaN_3
Secondary antibody solution	1%BSA in 1xTBS-Tween

Table 6. Kits

Name	Vendor	Catalogues No.
Click-iT® Plus EdU Alexa Fluor® 647 Flow Cytometry Assay Kit	Molecular Probes™	C10634
Pierce® BCA Protein Assay Kit	Thermo Scientific TM	#23225 #23227

Table 7. Cell culture reagents

Name	Vendor	Catalogue No.
A/C Heterodimerizer	Clontech	635057
Accutase	Sigma-Aldrich Co. LLC.	A6964
Collagen	Sigma-Aldrich Chemie GmbH	C7661
DMEM	Sigma-Aldrich Co. LLC.	D6429
DMSO	Sigma-Aldrich Chemie GmbH	D2650
EGF	Peprotech	AF-100-15
FCS	Biowest LLC	S1810-050
GlutaMax	Gibco® by Life technologies	35050-038
HEPES	AppliChem GmbH	A1069,0500
LY294002	Enzo Life Sciences	BML-ST420
MEM Amino acids	PAA Laboratories GmbH	11140-035

Nocodazole	Sigma-Aldrich Chemie GmbH	M1404
OptiMEM medium	Invitrogen, life technologies,	31985-070
Penicillin – Streptomycin	Sigma-Aldrich Chemie GmbH	P0781
Polyethylenimine (PEI) branched	Sigma-Aldrich Chemie GmbH	40,872,7
Puromycin	Sigma-Aldrich Chemie GmbH	P8833
RO-3306	Sigma-Aldrich Chemie GmbH	SML0569
Sodium pyruvate	PAA Laboratories GmbH	S11-003
Thymidine	Sigma-Aldrich Chemie GmbH	T1895
Trypsin-EDTA	Gibco®by Life technologies	25300-045
U0126 (MEK inhibitor)	Enzo Life Sciences	BML-EI282

Table 8. Primary antibodies and conjugates

Name	**Vendor**	**Catalogue No.**	**Application**	**Dilution**
Akt	Cell Signaling Technology®	9272	Western blot	1:2000
Anti-pan-Ras C-4	CALBIOCHEM®	sc-166691	Western blot	1:500
Cyclin D1 (92G2)	Cell Signaling Technology®	2978	Western blot	1:1000
EGF receptor	Cell Signaling Technology®	4267	Western blot	1:2000
H-Ras (C-20)	Santa Cruz Biotechnology®	sc-520	Western blot	1:1000
K-Ras (F234)	Santa Cruz Biotechnology®	sc-30	Western blot	1:1000

mTOR (human FRB Domain)	Enzo® Life Sciences GmbH	ALX-215-065	Western blot	1:1000
N-Ras (F155)	Santa Cruz Biotechnology®	sc-31	Western blot	1:500
p44/42 MAPK (ERK1/2) (137F5)	Cell Signaling Technology®	4695	Western blot	1:1000
p-EGF Receptor (Y1068)	Cell Signaling Technology®	2236	Western blot	1:1000
pErk (E-4)	Santa Cruz Biotechnology®	sc-7383	Western blot	1:1000
phospho-Akt (S473)	Cell Signaling Technology®	9271	Western blot	1:2000
phospho-Akt (Thr308)	Cell Signaling Technology®	9275S	Western blot	1:1000
phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) (Alexa Fluor® 647 Conjugate)	Cell Signaling Technology®	4375	Flow cytometry	1:50
RalA	BD Transduction Laboratories™	610221	Western blot	1:1000
Rap1	BD Transduction Laboratories™	610195	Western blot	1:1000
RB2 Clone 10	BD Transduction Laboratories™	610261	Western blot	1:1000
α-GFP	Ian A. Prior, University of Liverpool		Western blot	1:1000
α-RFP	Ian A. Prior, University of Liverpool		Western blot	1:1000

Table 9. Secondary antibodies

Antibody	Source	Dilution	Application	Vendor
HRP-anti mouse	goat polyclonal	1:10000	Western Blotting	KPL®
HRP-anti rabbit	goat polyclonal	1:10000	Western Blotting	KPL®
HRP-anti sheep	rabbit polyclonal	1:10000	Western Blotting	Santa Cruz Biotechnology®

Table 10. General chemicals

Name	Vendor	Catalogue No.
2-Mercaptoethanol	Fluka Biochemika	63690
4% Glutathione Agarose	Jena Bioscience	AC-210-CSTM
Acetic acid	Carl Roth GmbH & Co. KG	3738.5
Ammonium persulphate (APS)	SERVA Electrophoresis GmbH	13375
bisBenzimide H 33342 trihydrochloride (Hoechst33342)	Sigma-Aldrich Chemie GmbH	B2261
Bovine serum albumin (BSA)	PAA Laboratories GmbH	K41-001
Bromophenol blue	Carl Roth GmbH & Co. KG	A512.1
Calcium chloride ($CaCl_2$)	AppliChem GmbH	A4689,0250
Coomassie Brilliant Blue G-250	Carl Roth GmbH & Co. KG Fluka Biochemika	9598.1 27815
DAPI	Molecular Probes Invitrogen,	D1306
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH	D2650
Dithiothreitol (DTT)	AppliChem GmbH	A2948,0010
EdU (5-Ethynyl-2'-deoxy-uridine)	Jena Bioscience	CLK-N001
Ethanol	Carl Roth GmbH & Co. KG	K928
Ethylene glycol tetraacetic acid (EGTA)	AppliChem GmbH	A0878,0025

Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG	8043.2
Glycerol	Carl Roth GmbH & Co. KG	3783.2
Glycine PUFFERAN®	Carl Roth GmbH & Co. KG	3908.3
GST–Raf-1-RBD	In house	
GST-RalBD	In house	
Guanosine diphosphate (GDP)	Sigma-Aldrich Chemie GmbH	
Isopropanol	Carl Roth GmbH & Co. KG	6752.4
Leupeptin-hemisulphate	AppliChem GmbH	A2183, 0025
Magnesium chloride (MgCl2)	Carl Roth GmbH & Co. KG	HN03.1
Methanol	Carl Roth GmbH & Co. KG	3880.2
Microycystin-LR	Enzo Life Sciences GmbH	
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Serva Electrophoresis GmbH	35930
Nonylphenylpolyethylenglycol (Pietenpol and Stewart)-40	Merck KGaA	492016
PageRuler Prestained Protein Ladder	Thermo Fisher Scientific Inc.	26616
Paraformaldehyde	Sigma-Aldrich Chemie GmbH	158127
Pefabloc SC®(AEBSF hydrochloride)	AppliChem GmbH	A1421,0500
Pepstatin A	AppliChem GmbH	A2205,0025
Phenylmethylsulfonylfluoride (PMSF)	Sigma-Aldrich Chemie GmbH	P7626
Picolyl azide sulfo-Cy5	Jena Bioscience	CLK-1177
Potassium acetate	Carl Roth GmbH & Co. KG	4986.1
Potassium chloride (KCl)	Carl Roth GmbH & Co. KG	6781.1
Propidium Iodide	EMD Chemicals, Inc.	537059
RNase A from bovine pancreas	Roche Diagnostics GmbH	70297721
Rotiphorese® Gel 30 30 % Acrylamide/Bis-acrylamide (37.5:1)	Carl Roth GmbH & Co. KG	3029.1
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG	3957.2
Sodium Deoxy cholate	Sigma-Aldrich Chemie GmbH	D6750

Sodium Deoxycholate	Sigma-Aldrich Chemie GmbH	D6750
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Chemie GmbH	A3942,1000
Sodium hydroxide (NaOH)	Sigma-Aldrich Chemie GmbH	9356.1
Sodium orthovanadate	Sigma-Aldrich Chemie GmbH	S6508
Sodium-β-glycerophosphate	SERVA Electrophoresis GmbH	
Tris ultrapure	AppliChem GmbH	A1086,5000
Triton® X-100	Carl Roth GmbH & Co. KG	6683.1
Tween® 20	SERVA Electrophoresis GmbH	3747.0
Western Lightning ECL, Enhanced Chemiluminescent Substrate	PerkinElmer	0RT2655 0RT2755
Whatman filter paper	Bio-Rad Laboratories Inc.	

3.2. Methods

3.2.1 Cell lines and treatments

Cervical cancer (HeLa) cells, Human Embryonic Kidney 293 T (HEK293T) cells were originally provided by Yan Cui, Leibniz Institute on Aging – Fritz Lipmann Institute (Capon, Seeburg *et al.*), Jena, Germany. HEK293T cells (kindly provided by Yan Cui, FLI, Jena, Germany) DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) supplemented with 10% FCS. Stably transduced HeLa#1#7 cells generated from the former master student, Stephanie Bresan, were cultured at 95% humidity, 37°C and 5% CO_2 atmosphere in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FCS.

T98G glioblastoma cells kindly provided by Dr. Helmut Pospiech (FLI, Jena, Germany), were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FCS, 1x L-Glutamax, 1mM Sodium pyruvate and 1x NEAAs (non-essential amino acids) under standard conditions (95% humidity, 5% CO_2, 37°C). Cells were split 3-4 times per week when they reached 80% confluency.

Synchronized cells (see section 5) were treated with the heterodimerizer or inhibitors as indicated in the results section. In the cases that the samples were prepared for western blot analysis, the release media was supplemented with 10ng/ml EFG. In case the release occurred in the presence of the heterodimerizer or inhibitors, the latter was added to the cells starting from the last 30 min of the synchronization.

3.2.2. Transfection procedure

3.2.2.1 Transient transfection

Transient expression of the heterodimerization units within cells was achieved using polyethylenimine (PEI), a stable cationic polymer, as a transfection reagent. One day before being transfected, cells were seeded in a 6-well plate or glass-bottomed dish depending on whether they would be harvested for western blot or used for confocal microscopy experiments. The transfection is efficient if performed at a cell density of 50-60% confluency. Via a serial dilution in pre-warmed reduced serum media OptiMEM (1:20 and 1:25), a PEI solution was prepared with a final concentration of 20ng/ml. In parallel, 1-2µg of plasmid DNA was dissolved in 250µl of OptiMEM. In the next step, an equal volume of the PEI and DNA

solution (250µl each) were mixed and allowed to complex for 20-30 minutes at room temperature. It is important to mention that PEI must be added to the DNA solution and not the other way around. During this time, the media of the cell was replaced with 1ml of OptiMEM. After the incubation time is over, 500µl of PEI/DNA mixture were added to each well while gently swirling the plate by a slow hand rotation. Plates were incubated under standard conditions and after 4-6 hours media was exchanged with 2ml of DMEM with 10% FCS. The next day (24 hours post transfection) cells were checked under the microscope for GFP or mCherry expression and afterwards proceeded for western blotting or LSM measurements.

3.2.2.2 Lentiviral transduction

The stable expression of our constructs of interest in HeLa or T98G cell lines was achieved through a lentiviral transduction using the third-generation of lentiviral vectors. In this case, our plasmid DNA introduced in a lentiviral expression vector (pCDH) was co-transfected with two packaging vectors (pMDL-g and pRSV-g) and an envelope vector pVSV-g.

Production of the infectious lentiviral particles was performed in HEK293T cells. The latter were seeded in 100mm dishes at a density of 40-50% confluency (1.5-2 x 10^6 cells/dish). The next day a PEI transfection was performed on HEK293T cells, but with a modified DNA mixture containing 4 plasmids. Taking into account the limited amount of DNA that can be introduced in one single cell, the correct ratio between the four plasmids is of critical importance. Following this argument, the DNA solution for one reaction contained 8µg of expression vector and 14µg of lentiviral vectors mix dissolved in 1.5ml of OptiMEM. The ratio between pVSV-g, pRSV-g and pMDL-g was 1:2.4:3.6. After 25-30 minutes of incubation at room temperature, the PEI/DNA mixture was distributed at 3ml per dish and placed back in the incubator. The following procedure is the same as described in the previous section.

The same day, the target cells (HeLa or T98G) were seeded in 6-well plates at 30% confluency. The first transduction of the lentiviral particles was performed 24-48 hours after HEK293T cells were transfected. The supernatant of the transfected HEK393T cells was collected and filtered through a 0.4µM filter. Afterwards the viral particles were concentrated using Amicon Ultra-15 centrifugal filters via a centrifugation step of 10-13 minutes at 4000xg, at room temperature. The concentrated viral particles were mixed with 1x polybrene (8µg/ml) and

then equally distributed in the 6-well plate. As a final step the target cells underwent a centrifugation round for 1 hour at 500xg, at room temperature. The transduction step was repeated up to three times every 24 hours while cells were kept under standard conditions. After 24 hours from the last transduction step, cells were selected with puromycin for one week. Only cells that had efficiently incorporated in their genome our gene of interest would be resistant to the antibiotic. Depending on the cell line, the final concentration of puromycin in the media was 2µg/ml for HeLa cells and 1.5µg/ml for T98G cells. However, antibiotic selection is not enough to have a pure culture with only double positive cells. This is due to the gain of antibiotic-resistance that cells develop after some time of being exposed to relatively low concentrations of the antibiotic. The concentration used is high enough to induce apoptosis in not transduced cells and to allow survival of the transduced one.

3.2.3. Generation of homogeneous cultures

3.2.3.1 FACS Sorting of double positive cells

FACS sorting of the double positive cells was done with the help of Yvonne Schlenker, at the FACS facility at the Uniklinikum Jena. Cells were washed once with 5ml PBS and then detached from the flask with 2ml Accutase, a more gentle solution for cells than Trypsin, for 2-4 minutes of incubation under standard conditions. Cells were further mixed with 5-10ml of PBS, spinned down for 5 min at 300xg and re-suspended in 3ml of PBS supplemented with 10% FCS. Preparation of suspended cells was done shortly before being sorted. Three controls were required for correct gating of the double positive cells: wild type cells and two single positive cells each expressing one of our constructs, the GFP- or mCherry-bound unit. Sorted cells first collected in growth media, were washed once with PBS + 10% FCS before being transferred in a T25cm^2 flask. They were kept in the presence of antibiotics, Penicillin-Streptomycin, for few days after sorting to avoid contaminations.

3.2.3.2. Limiting dilution and clonal cultures

Sorted T98G#1#7 cells were trypsinized, counted using a Neubauer chamber and further diluted to 1-2 cells/ml. They were seeded in 96-well plate and followed for several weeks. Media containing 1.5µg/ml puromycin was renewed every 2-3 days. Before cells reached 90%

confluency they were transferred into 12-well plates and later on in T25cm^2 flasks. Meanwhile they were also checked for the presence of the constructs under the microscope.

3.2.4. Laser scanning microscopy (LSM)

Confocal laser scanning microscopy is a microscopic technique that provides images with high contrast and resolution by scanning the sample point by point or at multiple points at a time. The instrument has the ability to assemble the information only from an in-focus plane and rule out any signal coming from out-of-focus planes.

In order to obtain high quality images, cells were seeded in glass-bottomed dishes that were previously coated with collagen. Only the glass part of the dish was covered with sterile collagen solution (100µg/ml) and incubated for 1-2 hours at room temperature. After the collagen was recollected, the glass-bottomed dishes were washed twice with sterile water and allowed to thaw for 2 hours at room temperature. Collagen-coated glass-bottomed dished could be stored at 4^{O}C.

To investigate the heterodimerizer-induced translocation of the effector unit toward the anchor unit, HeLa or T98G cells expressing both units were seeded in collagen-coated glass-bottomed dishes at 30% confluency. The next day media was exchanged with fresh growth medium supplemented with 25mM HEPES. Images were taken using Zeiss LSM 510 inverted laser scanning microscope (LSM) and C-Apochromat x63 water immersion objective lens. Excitations of the fluorophores was achieved with Ar 488nm and HeNe 543nm lasers for EGFP and mCherry respectively. Addition of the heterodimerizer was performed on stage, without touching the glass-bottomed dish and images were captured at the indicated times (see Results). Images were further process using ZEN software.

3.2.5. Synchronization techniques

Cell cycle synchronization techniques are used to emphasise the result of a specific treatment in cycling cells. In our case, cells were synchronized cells in 4 different moments of the cell cycle: in G0 (resting state/quiescent) phase, G1/S border, G2/M border and in mitosis (M phase).

T98G cells were induced to exit the cell cycle and enter G0 after 72 hours of serum starvation. 230˙000 cells per 60mm dish were seeded and the next day growth media was exchanged with starvation medium after washing them three times with DMEM without serum. The starvation medium was supplemented only with 0.15% FCS which is enough to keep cells alive and not die during the 72 hours of starvation. Stimulation of quiescent cells to re-enter cell cycle was achieved by replacing the starvation with release media. The latter is a 1:1 ratio combination of growth with conditioned medium.

G1/S synchrony of T98G cells (seeded 200˙000 cells/60mm dish) was obtained through 24 hours of treatment with 2mM thymidine. The presence of the latter in the cell blocks DNA synthesis by inhibiting a necessary enzyme for this process, ribonuclease reductase. As a result cells progress throughout G1 phase, but are not able to start S phase. Release in the cell cycle was possible after removing the inhibitor through three steps of washings with PBS and by adding complete release medium (1:1 ratio of growth and conditioned media).

A double block with thymidine and RO3306 induced accumulation of cells in late G2 phase. Seeded cells (200˙000 cells/60mm dish) were treated with 2mM thymidine for 24 hours. After being washed 3 times with PBS, cells were released for 3 hours in release media and further incubated with 10µM RO3306 for 14 hours. RO3306 is a quinolinyl thiozoline derivate that inhibits the formation of cyclinB-CDK1 complex by binding the latter and leading to a G2 phase block. Removal of the inhibitor followed by 3 washing steps with PBS and addition of the release media induced cell cycle progression.

Synchronization in mitosis of HeLa cells was achieved by a double block with thymidine and nocodazole. The procedure is almost the same as in the previous paragraph with the only difference that RO3306 is substituted with nocodazole (100mg/ml). Nocodazole inhibits microtubule polymerization and stops mitotic spindle formation. For the release of HeLa cells only normal growth media (DMEM + 10% FCS) was used.

3.2.6. Ras activity assay

A Ras pulldown assay was performed to detect GTP-loaded levels of the protein for each timepoint. The cell extract was prepared by lysing through scraping cells in ice-cold lysis buffer supplemented with protease inhibitors, phosphatase inhibitors, DTT, GDP and GST-RBD (Ras-binding domain of Raf 1) after being washed once with ice-cold PBS. Depending on the dish,

the amount of the lysis buffer used varied from 850µl to 1.3ml for a 60mm and 100mm dish respectively. The complex GTP-loaded Ras-GST-RBD is extracted from the whole cell lysate through glutathione-sepharose beads that are added to the cell lysate after 20 minutes of centrifugation at 4^{O}C at 13000rpm and incubated for at least 30 min at 4^{O}C with mild rotation. Only for experiments performed in HeLa cells, the precipitate at this stage was dissolved in 50µl of 2x Laemmli solution and boiled for 5 min at 95^{O}C. This is the nuclear extract that was used later to detect cyclinD1 expression. Following incubation, samples were briefly centrifuged (10 seconds at 13˙300rpm) and 400µl of supernatant mixed with 100µl of 5x Laemmli solution were transferred in new vials. This represents the total cell lysate samples, including the total load of Ras protein. The RasGTP-bound beads were sequentially washed twice with 750µl of lysis buffer in absence of inhibitors. Dissociation of the complex GTP-loaded Ras-GST-RBD was obtained after treatment of the samples with 40µl of 2x Laemmli solution. Samples were further processed for western blotting.

3.2.7. Preparation of whole-cell lysates using RIPA buffer

Treated T98G cells were washed once with ice-cold PBS and further lysed directly in ice-cold RIPA buffer, 850µl for 60mm dishes, to obtain cyclinD1 and pRB2 expression levels. Samples were cleared by spinning for 20 minutes at 13˙300rpm, at 4^{O}C and 800µl of the supernatant mixed with 200µl of 5x Laemmli buffer were transferred in new eppendorf tubes. From the remaining supernatant, 5µl were used for protein quantification. At this point, lysates can be stored at -20^{O}C or processed for western blotting.

3.2.8. Protein quantification

Protein concentrations were quantified using Pierce BCA Protein Assay Kit following the manufacturer's instructions. The assay was performed in a 96-well plate and the absorption measured using a spectrophotometer at 570nm. The final concentrations of the samples were calculated using a known standard BSA concentration range developed in parallel.

3.2.9. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in vertical electrophoresis system, using 40-70µg proteins. Depending on the molecular weight of the analysed protein, 7.5% and 12.5% polyacrylamide separating gels were used. Protein separation was achieved after approximately 3h of run in SDS buffer at a constant current of 20-40mA.

3.2.9.1. Western blotting and immunodetection of immobilized proteins

Except for the GST-Raf1-RBD whose visualization was through Coomassie staining, all other resolved proteins were transferred in activated 0.45µm or 0.20µm pore-size PVDF membranes (in ethanol-water) through semi-dry or wet transfer. Membranes were subsequently blocked for 30-45 minutes at room temperature with 1% BSA in TBS-Tween solution. Incubation with the primary antibody was performed overnight at $4^{O}C$ with mild shaking. After three consequent washes with 1xTBS-Tween for 10 minutes, membranes were incubated for 30-60 minutes with mouse or rabbit secondary antibody prepared in 1% BSA in TBS-Tween solution. A last round of washing step was followed by visualization of the peroxidase-coupled secondary antibody with Western Lighting PLUS-ECL Chemiluminescent Substrate and the signal was detected with the Fujifilm LAS-4000 documentation system. If required, membranes were stripped for 30 minutes at $50^{O}C$ under mild shaking using stripping buffer. All washing steps were also performed under soft agitation.

3.2.10. Flow cytometry

3.2.10.1. EdU incorporation Click-iT for cell cycle analysis

Cell cycle distribution of T98G cells was determined mainly through pulse-labelling of the cells with EdU (5-ethynyl-2'-deoxyuridine, a thymidine analogue) combined with DNA staining. The advantage of this technique is a more precise S phase quantification and is based on the copper-catalysed chemical reaction (Click-iT reaction) between the alkyne group of EdU and the azide-fluorophore (Alexa647 or Cy5) found in the Click-iT reaction cocktail (Fig. 7).

EdU-labelled cells (10µM for the last 10 min of treatment) were washed once with 1ml of 1x PBS and harvested with 800µl of pre-warmed Accutase. Suspended cells were washed once with 1ml of 1% BSA in PBS (further referred to as 1% BSA solution) and fixed sequentially with freshly prepared 4% PFA – 1% BSA (15 minutes of incubation in the dark, at room temperature) and ice-cold 100% ethanol. Ethanol addition was performed dropwise and while gently vortexing the sample to avoid cell clamps formation. At this stage, samples can be stored at -20^{O}C for up to 2 weeks. Removal of the fixative was achieved by centrifugation for 5 minutes at 300xg and one washing step with 1% BSA solution. Fixed cells were further permeabilized with 200µl of 0.5% Triton X-100 in 1% BSA or 100µl of saponin-based solution (provided by the Click-iT EdU-AlexaFluor647 Flow Cytometer Assay kit). Following 15 minutes of incubation protected from the light, samples were mixed carefully with 500µl of 1% BSA and incubated for another 20 minutes under the same conditions. Meanwhile, the click-iT reaction cocktail was prepared as shown in table 9. Removal of the permeabilization buffer by a centrifugation step for 5 minutes at 400xg is followed by a 30 minutes incubation with 100µl of the homemade kit or 500µl of the Click-iT EdU-AlexaFluor647 protected from the light. Samples were further washed once with 1% BSA, resuspended in DAPI/PBS solution (1µl DAPI from 40µg/ml stock in 300µl PBS) and analysed using the LRS Fortessa flow cytometer (BD Bioscience) equipped with blue (488nm) and red (633nm) lasers. In some experiments, the DAPI was substituted with an equivalent like Hoechst33342 or Propidium Iodide.

Table 11. EdU Click-iT reaction cocktail

A. Homemade kit

	1ml
Reaction buffer	860µl
100mM $CuSO_4$	40µl
100µM Picolyl-azide sulfo Cy5	10µl
20mg/ml Sodium ascorbate	100µl

B. Click-iT EdU-AlexaFluor647 kit

	x1 reaction
Click-iT reaction buffer	438µl
$CuSO_4$	10µl
Alexa Flour 647 azide	2,5µl
Reaction buffer additives	50µl

FlowJo software was used for the quantification analysis of all measurements. Gating of the total number of cells was done in a FSC-A vs SSC-A dot plot, from which a second dot plot DAPI-A vs DAPI-W was created to distinguish singlet nuclei from doublets or aggregates. These singlet nuclei are displayed in a third DAPI-A vs APC-A (EdU-Alexa647 or EdU-Picolyl-azide sulfo Cy5) dot plot. Further gating of the distinct phases of the cell cycle is shown in figure 6.

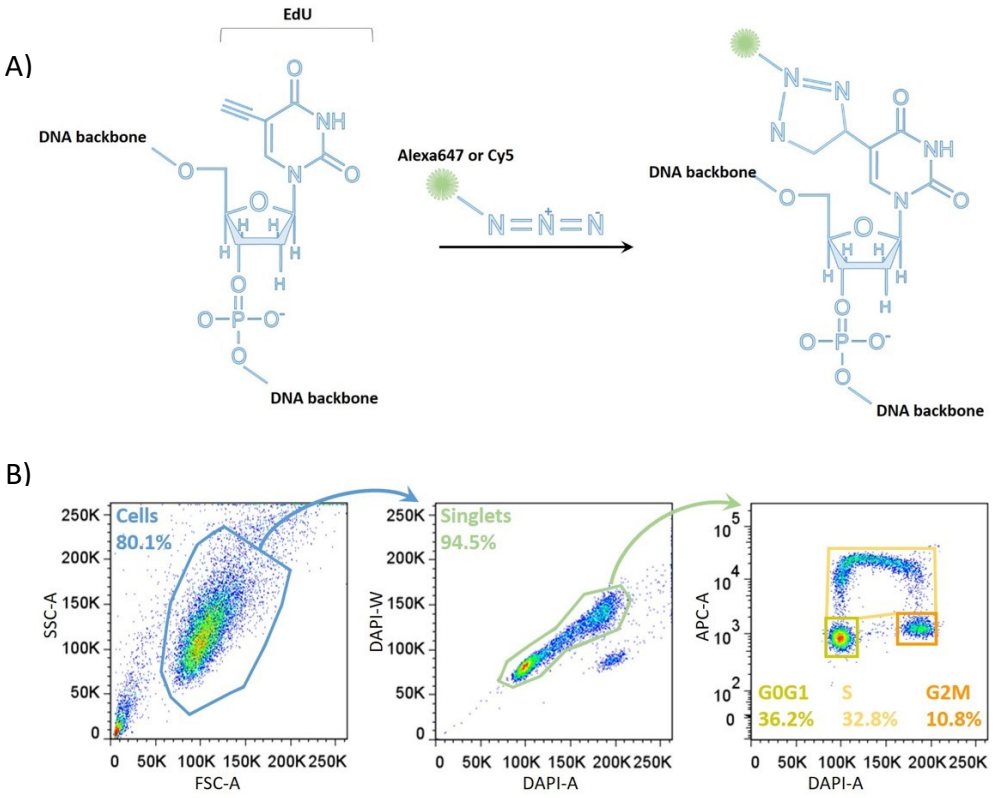

Figure 7. Cell cycle analysis using EdU-pulse incorporation.

A) Copper-catalysed reaction of the EdU-incorporated DNA backbone with the fluorophore-tagged picolyl-azide.

B) Sequential gating of singlet nuclei and distinct cell cycle phases.

3.2.10.2. Cell cycle analysis with PI staining

After the corresponding treatment indicated form the specific experiment, cells were harvested with 800µl Accutase, washed twice with 1ml and 3ml 1x PBS and resuspended in 300µl of ice-cold PBS. Samples were fixed with 750µl ice-cold 100% ethanol under gentle vortexing. Ethanol fixed samples can be stored up to 2 weeks at -20^{O}C. For cell permeabilization, 20-30 minutes incubation on ice with saponin-based or 0.5% Triton X-100 buffers was performed. Following 5 minutes of centrifugation at 500xg, cell pellet was resuspended in 100µl of RNaseA solution with a final concentration of 200µg/ml and

incubated for another 20 minutes. After a final centrifugation step (5 min at 400xg), cells were stained with 300µl of PI/PBS buffer (1.5µg/ml final concentration) and analysed on a FACS Canto (BD Bioscience) flow cytometer equipped with 633nm red laser. Gating of the total amount of cell and singlets was performed in the same way as described in the previous section. Cell cycle distribution of the singlet nuclei is displayed in a histogram of propidium iodide-A (Fig. 8A).

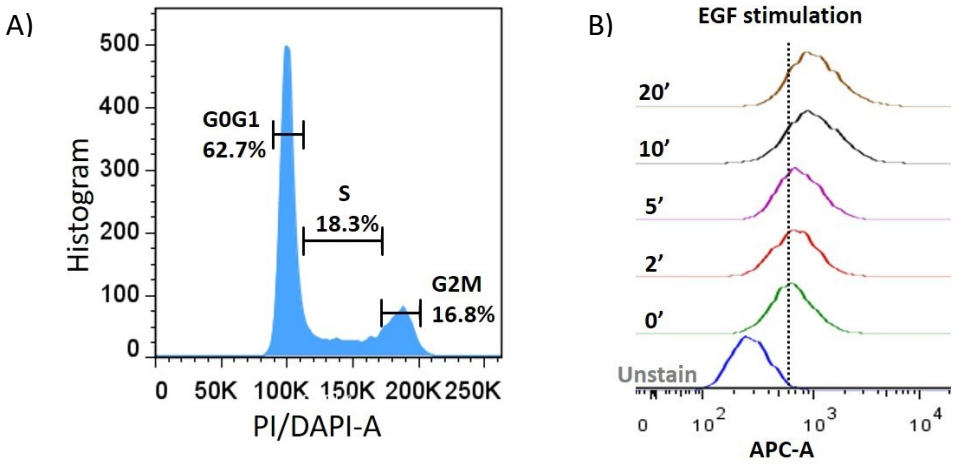

Figure 8. Cell cycle distribution and protein activation.

A) Representative gating of the cell cycle phases in a propidium iodide histogram. B) Measurement of Erk protein activation using an Alexa647-tagged antibody.

3.2.10.3. Detection of Erk phosphorylation by flow cytometry

After EGF stimulation, cells in culture dishes were PBS-washed, harvested, fixed and permeabilized as described in section 7.1. Permeabilized cells were blocked for 30 minutes with 5% donkey serum / 1% BSA / 1x saponin in PBS and further stained with Alexa647-conjugated Erk1/2 (pT202/pY204) for 1.5 hours protected from the light. PBS-washed cells were resuspended in 300µl PBS and analysed on the LRS Fortessa flow cytometer (BD Bioscience) using the red (633nm) lasers. Following the subsequent gating of the cells and singlet nuclei, the latter was plotted in an APC-A histogram (Fig. 8B).

3.2.11. Two step cell cycle analysis with NucleoCounter® NC-3000™

The NucleoCounter® NC-3000™ system enables a rapid quantification of DNA content by image analysis of the samples. After treatment, cells were harvested with pre-warmed

Accutase, PBS-washed and thoroughly resuspended in 250µl lysis buffer supplemented with 10µg/ml DAPI. After 5 minutes incubation at room temperature, cells were mixed with 250µl stabilization buffer and loaded into the 2-chamber slide (30µl). The loaded slide is further placed on the tray of the instrument where the quantified cellular fluorescence is displayed in a DAPI histogram. All solutions used in this protocol: lysis, stabilization and DAPI buffer were provided by the company.

4. Results

4.1. Characterisation of the heterodimerizer-induced effect

4.1.1. Expression of the anchor and effector units within cells

The heterodimerization system presented in this thesis is composed of two fusion proteins (anchor and effector units) and was partially characterized by a former master student, Stephanie Bresan.

The design of the two-unit heterodimerization system incorporated the generation of fusion proteins (anchor unit with FKBP/Ras-hvr and effector unit with FRB/NF1) in various permutations so as to maximise the chances of effective membrane anchorage of the anchor unit and efficient membrane recruitment of the effector unit upon addition of the stimulus. As depicted in figure 9, several combinations of the candidate anchor-effector unit pairs were tested.

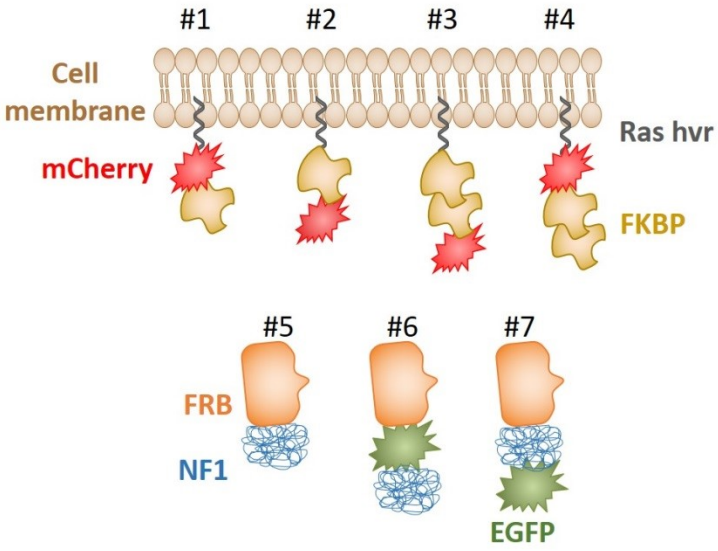

Figure 9. Constructs for the inducible heterodimerization systems.

The anchor units contains the dimerization domain FKBP fused to K-Ras-hvr and a fluorescent protein mCherry. The effector unit is composed from the dimerization binding partner FRB coupled to a RasGAP NF1 and to the fluorescent protein, EGFP.

Four anchor units were initially cloned, of which two had a single FKBP domain (designated #1 and #2) and two others contained two tandem FKBP domains (designated #3 and #4). By

design, all anchor units were tagged with a red fluorescent dye mCherry to visualise expression in a live cells. Along similar lines, three effector units, varying in their domain orientation, were marked by a green fluorescent dye EGFP (Enhanced Green Fluorescent Protein).

All possible combinations of the candidate anchor and effector units were analysed for their expression levels, localisation and membrane recruitment in transfected cells. First, transiently transfected HeLa cells revealed a different expression of the fusion proteins where the anchor units #2 and #3 had the strongest expression, followed by #1 and #4. Among the effector units, #7 was weakly expressed whereas construct #6 showed the strongest signal. Construct #5, which lacked the EGFP tag, could not be detected using the GFP antibody (Fig. 10).

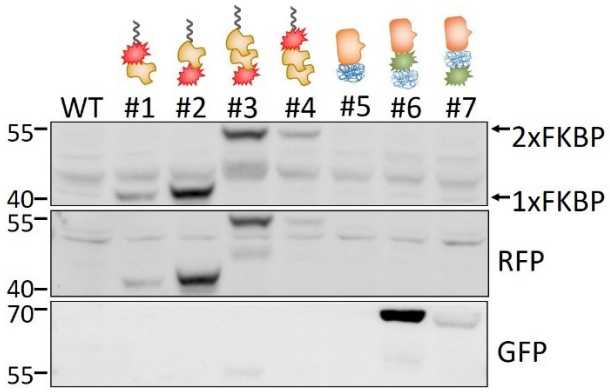

Figure 10. Expression of the anchor and effector units in HeLa cells.

HeLa cells were transiently transfected with each one of the constructs using PEI as a transfection reagent. The next day, cells were lysed and expression of the effector and anchor units was tested using specific antibodies. The anchor unit was detected with anti-FKPB and anti-RFP (red fluorescent protein) antibodies. Whereas to identify the anchor unit, anti-GFP antibody was used. As a negative control served wild type HeLa. Experiment performed by S. Bresan.

In the next step, all detectable constructs were analysed for their localization and membrane translocation in response to heterodimerizer-addition, a cell permeable inert rapamycin analogue, that results in the coupling of the FKBP and FRB domains. Confocal microscopy experiments in cells co-transfected with several combinations of anchor-effector units confirmed correct intracellular localization; the anchor units were stably bound to the plasma membrane while the effector units were detected by diffuse, green cytoplasmic fluorescence.

Upon heterodimerizer addition, the GFP-coupled effector unit was observed to translocate towards the mCherry-coupled membrane-bound unit, evident within the first 15 minutes of heterodimerizer treatment. Two candidate pairs, #1#7 and #4#7 respectively, showed promising results for use as a system in HeLa and MEF, respectively (Fig. 11). The chosen anchor-effector pairs were then, stably introduced into HeLa cells by means of lentiviral transfection, designated HeLa#1#7 and HeLa#4#7.

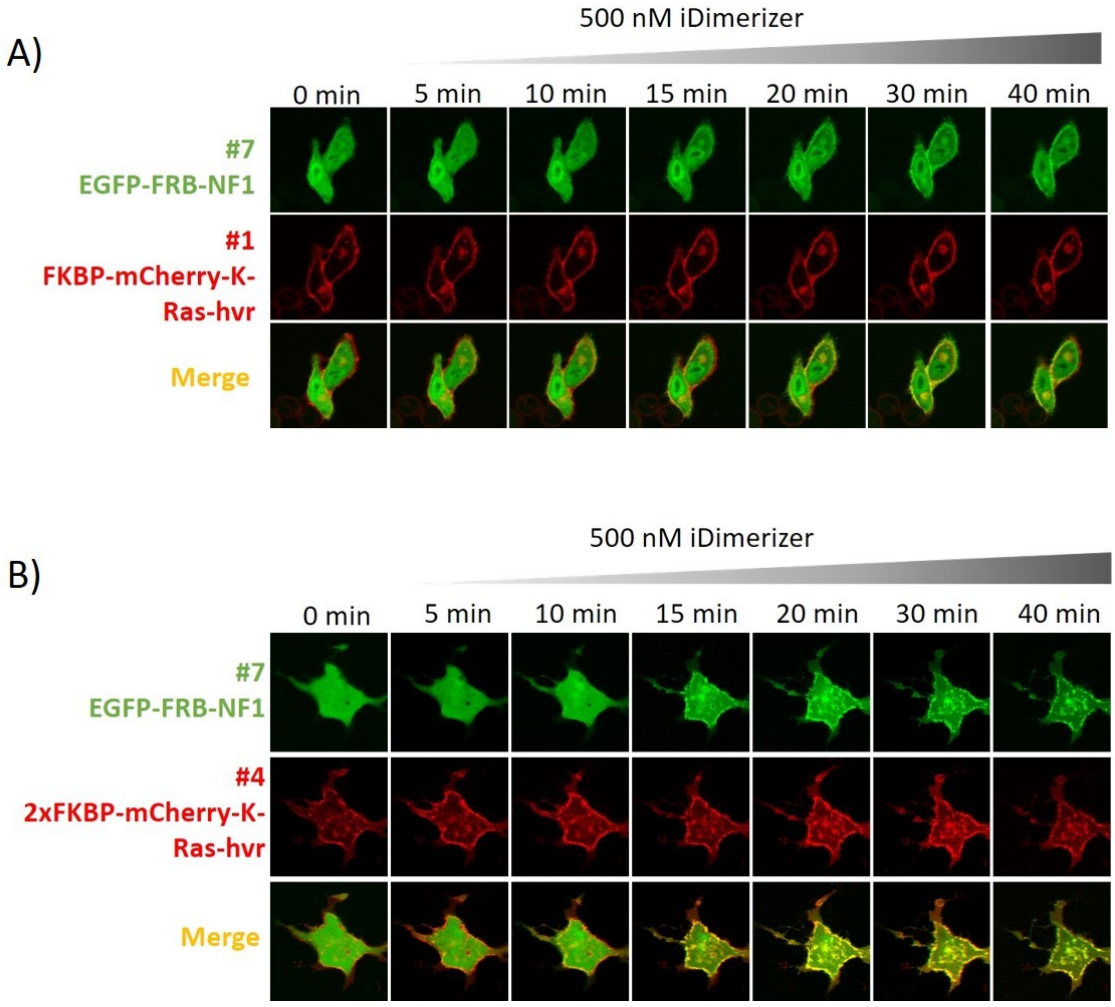

Figure 11. Induced translocation of the effector unit (EGFP-FRB-NF1) toward the anchor unit (FKBP/2xFKBP-mCherry-K-Ras-hvr).

A) HeLa cells transduced with the anchor and effector unit, #1 and #7 respectively, were incubated with 500nM heterodimerizer (iDimerizer) up to 40 minutes. Confocal images were taken prior (0 min) and after heterodimerizer addition at the indicated time points. B) Same procedure was performed in MEFs transfected with constructs #4 and #7. Experiment performed by S. Bresan.

4.1.2. Dose- and time-dependence effects of the heterodimerizer

Since the response-time of the system varies with the concentration of heterodimerizer stimulus used (Inoue, Heo *et al.* 2005), we sought to determine the conditions best suited for our experiments by performing a dose response analysis. Serum-starved HeLa#1#7 cells were treated for 1 hour with 5nM, 50nM and 500nM heterodimerizer and then stimulated with 10ng/ml epidermal growth factor (EGF) to follow Ras activation in time. The results of the Ras activity assay thus obtained are presented in figure 12. While EGF induced a rapid activation of Ras protein (collectively referring to N-/H-/K-Ras) and its direct downstream effector Erk in untreated HeLa#1#7 cells, Ras activity declined (as measured by GTP-bound Ras levels) in all the tested heterodimerizer concentrations, with the highest dose strongly inhibiting Erk phosphorylation. Based on these results, 500nM heterodimerizer was used in subsequent experiments.

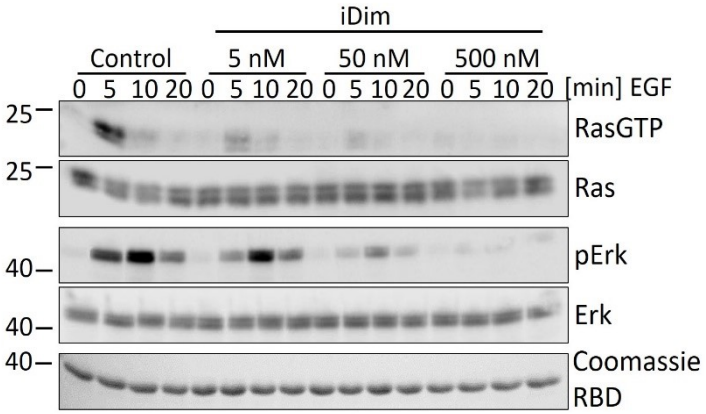

Figure 12. . High concentration of the heterodimerizer induced the higher decline in Ras and Erk activity.
Dose-response westerns of previously starved HeLa#1#7 treated for 1 hour with the indicated dose-range of heterodimerizer. Following stimulation with 10ng/ml EGF at the indicated time points, Ras activity was determined with a RasGTP pulldown assay. The double band detected from specific anti-Ras antibody correspond to K-Ras (upper band) and N-Ras/H-Ras (lower band. Erk phosphorylation levels (pErk) were determined using phosphosite-selective antibody. Coomassie RBD: coomassie stain of the Ras binding domain used to selectively separate active Ras (RasGTP) from the inactive form. Coomassie staining served as a loading control. Experiment performed by S. Bresan.

A second important factor under consideration was the stability of the heterodimerizer in culture. We tested serum-starved HeLa#1#7 cells kept in the presence of the heterodimerizer for longer periods of up to 16 hours in Ras activity assays after EGF stimulation. All treated

cells showed no Ras activity and a strong inhibition of Erk phosphorylation, indicating a long-term stability of the heterodimerizer (Fig. 13).

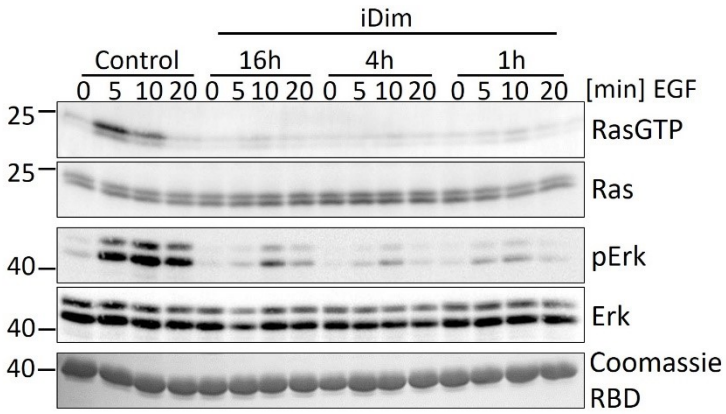

Figure 13. Long-term abrogation of Ras and Erk activity upon heterodimerizer treatment.
Overnight starved HeLa#1#7 were kept in the presence of 500nM heterodimerizer for 1, 4 and 16 hours and further re-stimulated with 10ng/ml EGF at the indicated time points. Ras and Erk activity were detected by a Ras activity assay and western blot analysis. Coomassie staining served as a loading control. Experiment performed by S. Bresan.

4.1.3. The heterodimerization system is Ras-specific

The heterodimerization system was constructed with the intent of targeting Ras specifically, without interfering with Ras signalling cascade at any other level. In order to verify this, we monitored upstream and downstream proteins in the Ras pathway in EGF-stimulated, serum-starved HeLa#1#7 cells in the presence or absence of heterodimerizer. Wild-type HeLa cells lacking the dimerization system were included as a biological control (Fig. 14). Note that while EGF receptor phosphorylation, and hence its activation, was comparable in wild type HeLa, untreated HeLa#1#7 and heterodimerizer-treated HeLa#1#7 despite lower total EGFR levels in the latter two, EGF-induced Ras activity and Erk phosphorylation levels were significantly reduced in heterodimerizer-treated HeLa#1#7. Since PI3K pathway can be modulated by Ras, Akt activity downstream of PI3K was examined. Heterodimerizer-induced Ras inhibition was also evident from a decline in Akt phosphorylation.

The apparent difference in Ras activation between wild type HeLa and HeLa#1#7 may be attributed to an overexpression of RasGAP (NF1) and/or generally weaker Ras and EGF receptor expression in these cells.

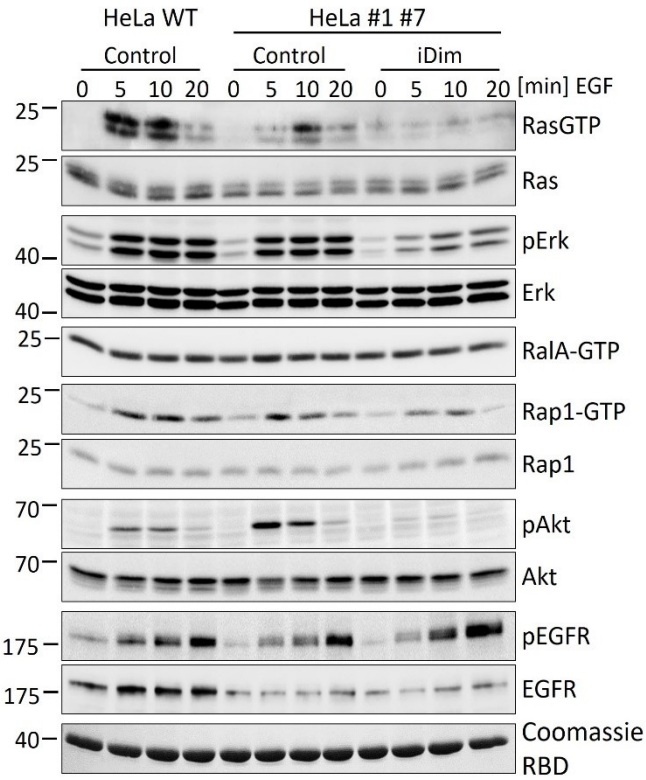

Figure 14. The RasOFF system is highly specific to Ras protein.

Immunoblots analysis to determine Ras activity and its interaction with downstream effectors. Wild type HeLa cells served as behaviour control for HeLa cells stably expressing the RasOFF system. Cells were starved overnight and then stimulated with 10ng/ml EGF in absence or presence (1 hour) of 500nM heterodimerizer at the indicated time points. Representative immunoblots of RasGTP levels, its downstream (Erk, Akt) effectors and upstream proteins (EGFR). Activity of two related small GTPases (RalA and Rap1) were also determined. Coomassie staining served as a loading control. pAkt: detection of the phosphorylated Akt. pEGFR: detection of the phosphorylated EGFR. Experiment performed by S. Bresan.

In addition, the activity of two other small G proteins, Rap1 and RalA was investigated. Interestingly, only Rap1 was stimulated by EGF, while RalA showed a steady basal activity. Heterodimerizer addition did not alter Rap1 and RalA activities. In case of HeLa#4#7, Ras activity was not inhibited upon heterodimerizer treatment, suggesting that the physical binding of the anchor and effector unit does not necessary lead to a biological function of the system (data not shown).

4.2. General strategy to study the role of Ras in G0-G1-S transition

The inducible heterodimerization system was employed to study importance of Ras in cell cycle entry and progression by inhibiting its activity in cells emerging from quiescence. Two essential prerequisites dictated our choice of the correct cell model. Firstly, cells must have an intact endogenous Ras activity. Secondly, they must be practically amenable to serum-starvation induced quiescence and serum-supplementation induced cell cycle re-entry. After several trials in an exhaustive list of primary cells such as BJ, Wi-38, MRC-5, IMR-90 and transformed cell lines like MCF-7, HeLa, HepG2, T98G, we inferred that the latter best fulfilled our requirements.

T98G cells are derived from a human glioblastoma multiforma tumour and can easily be made quiescent by serum withdrawal. The proliferation of these cells was reported to be dependent on the Ras-MAPK pathway (Szöőr, Ujlaky-Nagy *et al.* 2016) and no mutations in RAS genes have been reported. In spite of possessing certain characteristics of transformed-cells, like immortality and anchorage independence (Stein 1979), T98G cells maintained normal checkpoint mechanism for G1-arrest in response to serum withdrawal or contact inhibition. Moreover, when deprived of serum for 3 – 6 days (DMEM supplemented with 0.15% FCS), T98G cells exit the cell cycle to enter a G0 quiescent state (Rytkonen, Hillukkala *et al.* 2006).

To determine the shortest and most efficient synchronization method, we starved T98G cells for 3, 4, 5 and 6 days. Regardless of the starvation period, over 80% of cells were arrested in G0-G1 and the comparison of the cell cycle re-entry after serum stimulation showed no difference (Fig. 15). Therefore, in future experiments, T98G cells will be rendered quiescent by 72 hours of serum withdrawal.

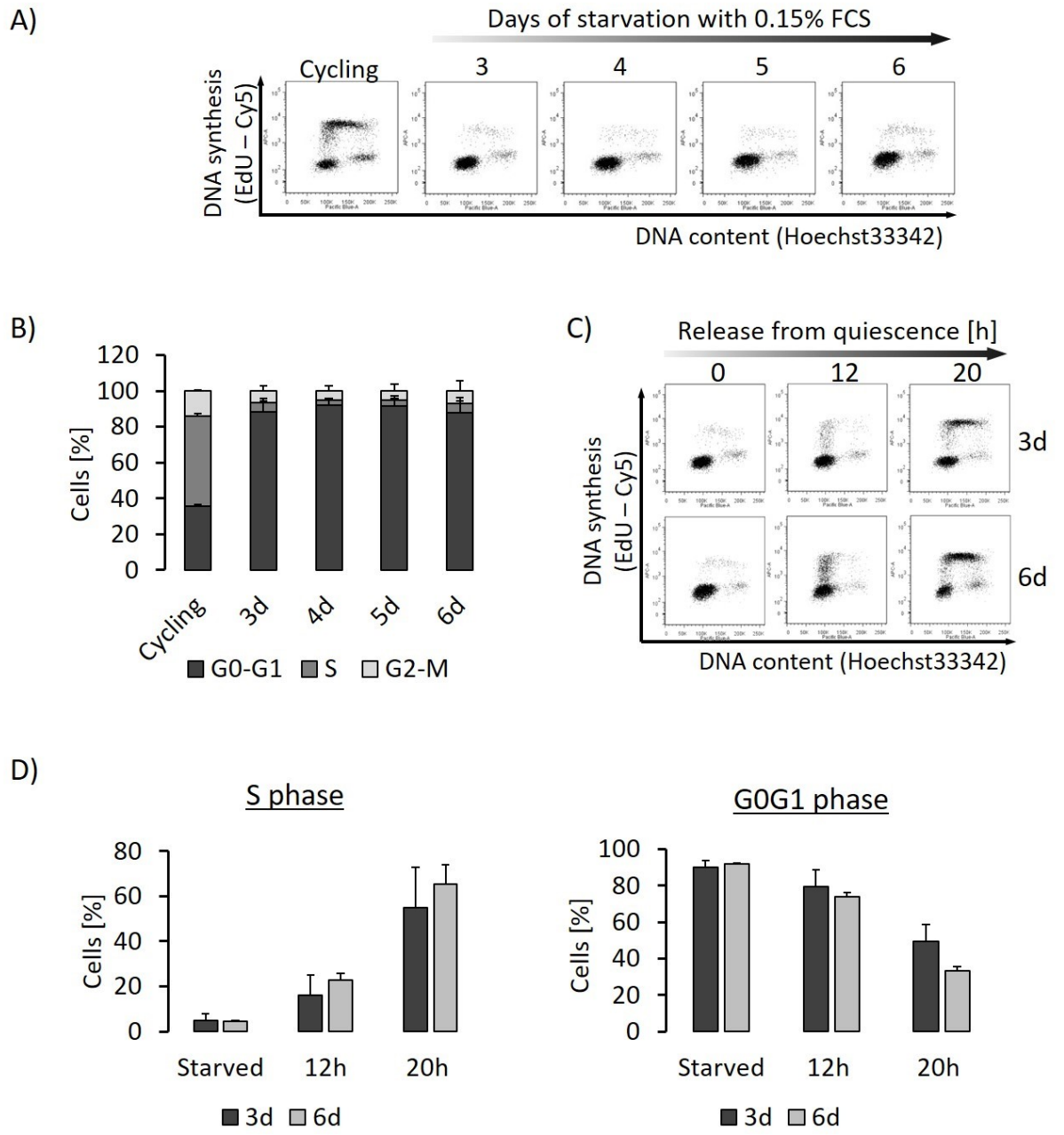

Figure 15. Comparison of the synchronization efficiency upon starvation and tracking of cell cycle re-entry.

A) T98G cells were induced to enter quiescence upon starvation for 3, 4, 5 and 6 days. T98G cells were kept in medium with only 0.15% FCS and then fixed for cell cycle analysis through EdU-pulse (10min) incorporation of newly synthesized DNA followed by a copper-dependent Click–iT chemistry based-detection. B) Quantification of cell cycle distribution of actively cycling and starved cells. C) Tracking of cell cycle re-entry and progression up to 20 hours upon FCS stimulation of quiescent T98G cells. D) Quantifications of G0G1 and S phase after 12 and 20 hours of release of T98G emerging from quiescence. Values are presented as mean ± SD of three independent experiments.

4.2.1. Generation of stable cell lines

The anchor and effector units of the heterodimerization system were simultaneously introduced in T98G cells through a lentiviral transfection. After 1 week of puromycin selection, a FACS-Sorting of the double positive cells was performed. Sorted T98G #1#7 were analysed for the physical presence of the constructs #1 and #7 within the cell through western blot analysis. As shown in figure 16A the overall expression of both constructs in our culture was stable.

As a second step, T98G#1#7 cells were used to test the functionality of the RasOFF heterodimerization system through a RasGTP pulldown assay. Previously starved T98G#1#7 cells were incubated with 500nM heterodimerizer for 1 hour and subsequently stimulated with EGF for the indicated time points (Fig. 16B). Upon EGF stimulation of the control set, Ras is activated and reached a peak within the first 5 minutes. Longer EGF stimulation shows a decline of its activity to a basal level. Treatment with the heterodimerizer induced reduction of RasGTP levels and a moderate Erk inactivation. Akt activity was unaltered. Under identical settings, the activity of the downstream Ras effector, Erk, was monitored by flow cytometry in one of the clones. Administration of the heterodimerizer induced a repression on Erk phosphorylation levels. Progressive rightward shift of histograms with time compared to the EGF-unstimulated sample in the control (Control) series indicate an increase in Erk activity, while reduced p-Erk signals (no rightward shift of peaks) in the treated (iDim) series reflect Erk is inactive (Fig. 16C).

During confocal microscopy experiments, we noticed a wide variety of the green fluorescent intensity (EGFP expression) emitted by cells, whereas only a small portion showed a bright EGFP signal. The opposite was observed for construct #1 FKBP-mCherry-K-Ras-hvr, of which T98G #1#7 cells displayed a very homogeneous mCherry (red) signal (Fig. 16D).

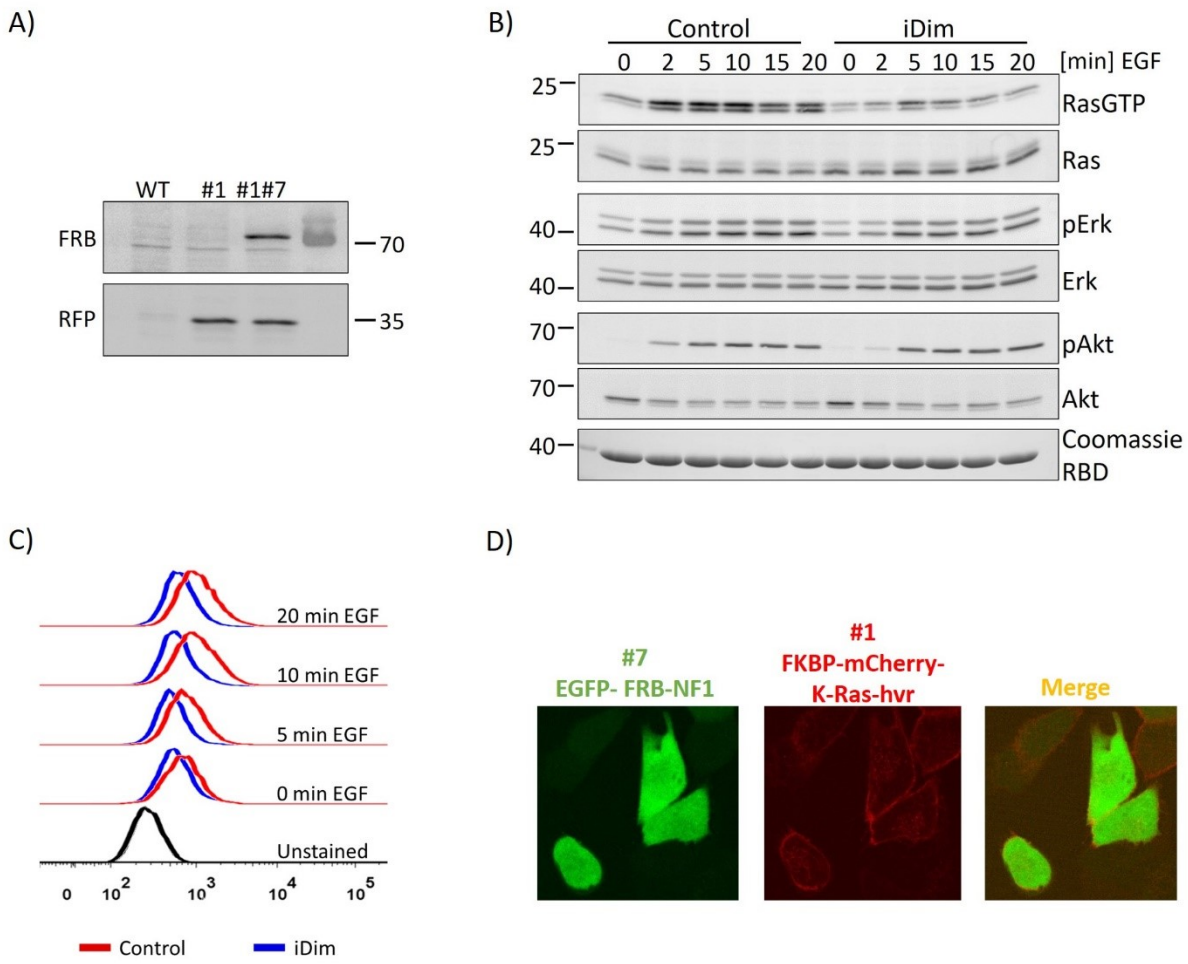

Figure 16. Transduced T98G #1#7 cells.

A) Transduced and sorted T98G#1#7 cells were tested for the expression of the effector and anchor unit through western blot analysis. Wild type T98G cells served as negative control, while T98G cells transduced only with the anchor unit served as single positive control. The presence of the #7 effector unit was detected with anti-FRB antibody, whereas the #1 anchor unit with anti-RFP antibody. B) Representative immunoblot (1 of n=2) of untreated and treated T98G#1#7 cells with heterodimerizer. Starved cells were incubated for 1 hour with 500nM heterodimerizer prior to EGF stimulation (10ng/ml). At the indicated time points samples were processed for RasGTP pulldown assay. Coomassie staining served as a loading control. C) Under identical conditions a flow cytometry analysis was performed to detect Erk phosphorylation levels. D) Confocal microscopy of T98G #1#7 cells. Note the heterogeneity of the #7 effector unit between cells, in contrast to the homogeneity of the #1 anchor unit.

4.3 Evaluation of Ras importance in cell cycle re-entry of quiescent cells

We continued our investigation by testing the cell cycle re-entry of quiescent T98G#1#7 cells. After 3 days of starvation with 0.15% FCS, cells were stimulated to enter the cell cycle in the absence or presence of the heterodimerizer. FACS analyses for the cell cycle distribution were performed after 12 and 16 hours of release (Fig. 17). Our initial results indicated a significant decrease or delay in the proportion of cells entering S phase when Ras activity was inhibited using the RasOFF system. These data confirm not only the requirement for Ras activity in cells emerging from quiescence as shown in the literature, but also the ability of our heterodimerization system to effectively attenuate Ras activity and consequently decrease S phase entry of serum-stimulated quiescent cells. The reduction of S phase entry can be due to the heterogeneity of T98G#1#7 culture observed during confocal microscopy experiments.

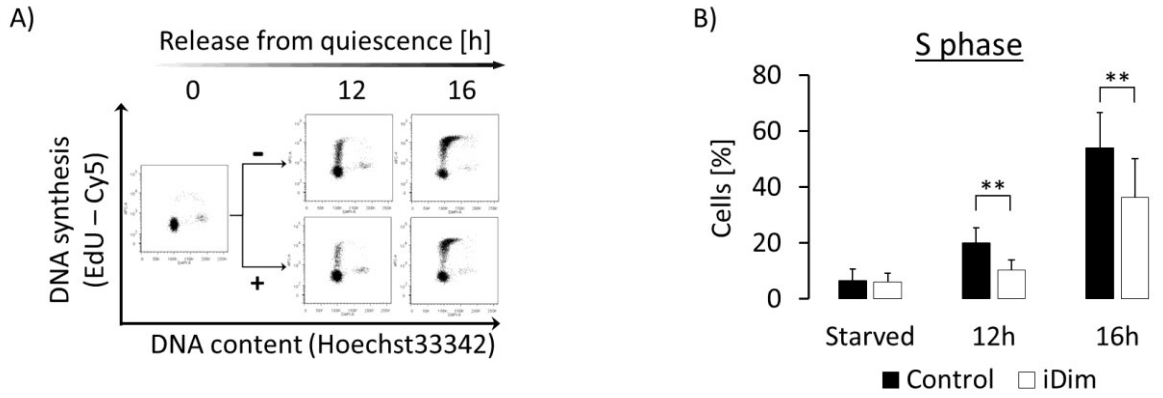

Figure 17. Determination of the cell cycle progression for T98G #1#7 cells emerging from quiescence.

A) T98G#1#7 cells were made quiescence after 72 hours of 0.15% FCS. Cell cycle re-entry upon serum stimulation was performed in the absence (-) and presence of 500nM heterodimerizer (+). At the indicated time points cell cycle distribution was determined through EdU incorporation in flow cytometry. B) Quantifications of S phase at the end of the synchronization (Starved), after 12 and 16 hours of release. Values are presented as mean ± SD of three independent experiments. Statistical significance was calculated using two-way ANOVA. **p<0.01, ***p<0.001

In order to ensure expression of the anchor and effector subunits in stoichiometric proportions to improve efficiency of the heterodimerization system, we resorted low- and high-EGFP cells from parental T98G#1#7 cultures, P4B/L and P6 respectively (Fig. 18), as well

as isolated single cell clones from them. Most of the single clone cultures expressed a weak, though homogenous EGFP signal (data not shown).

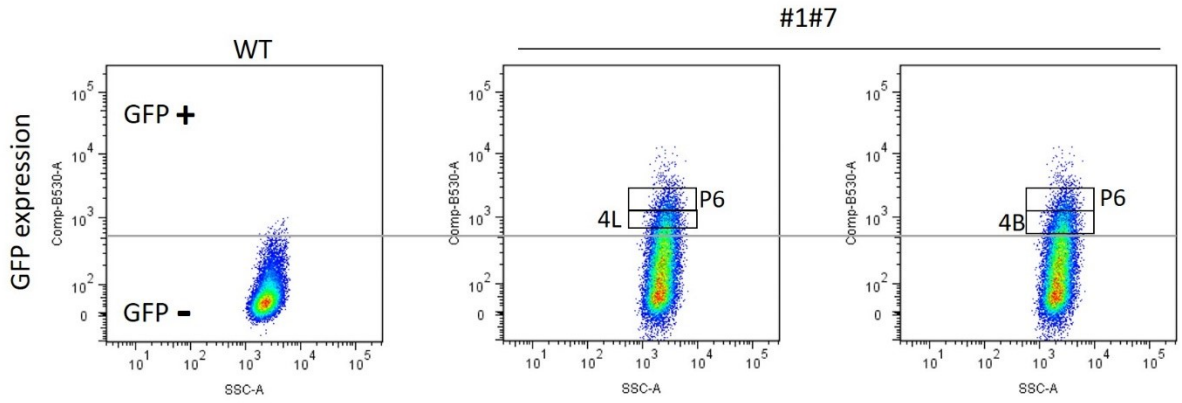

Figure 18. Representative gating strategies.

T98G#1#7 cells were first gated for mCherry expression and subsequently for similar EGFP intensity (populations P4B, P4L and P6). Wild type T98G cells were used as double negative control.

We proceeded by comparing Ras inhibition, upon heterodimerizer treatment, of the twice-sorted populations and the clones through Ras activity assays. Figure 19 shows a representative immunoblot for one of clones (G4) and one of the twice-sorted populations (P6). Overnight starved cells were treated with 500nM heterodimerizer for 1 hour and further stimulated with 10ng/ml EGF at the indicated time points. The heterodimerizer-induced Ras inhibition was observed in both cases, but the clone demonstrated a significantly higher effect on Ras. Decline in Erk phosphorylation was also more visible in the clone than the twice-sorted cells. Akt activity instead remained largely independent of Ras inhibition in both cultures.

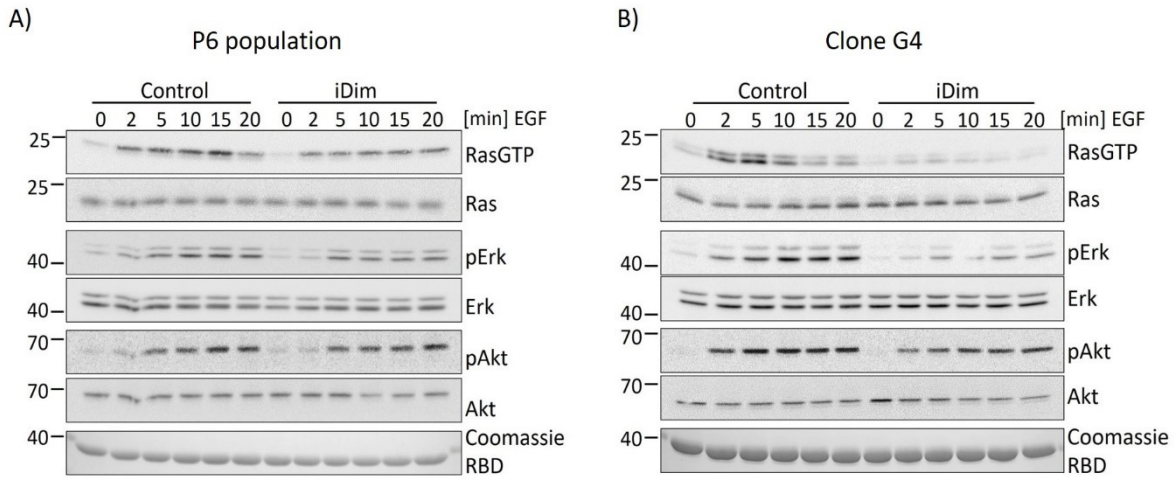

Figure 19. Heterodimerizer-mediated effect on Ras-GTP levels and its downstream effectors.

T89G#1#7 cells deprived of serum, treated or untreated with heterodimerizer for 1 hour were challenged for the indicated time frames with 10 ng/ml EGF. Representative immunoblot of Ras pulldown assay (1 of n=2) showing RasGTP levels and its downstream effector activities upon heterodimerizer addition of P6 population (A) and G4 clone (B). Coomassie staining served as a loading control.

For all the clones and populations, FACS analysis for the cell cycle distribution were also performed in cells emerging from quiescence in the absence and presence of the heterodimerizer. Twice-sorted populations showed a similar S phase entry like the parental T98G#1#7 cells (Fig. 20).

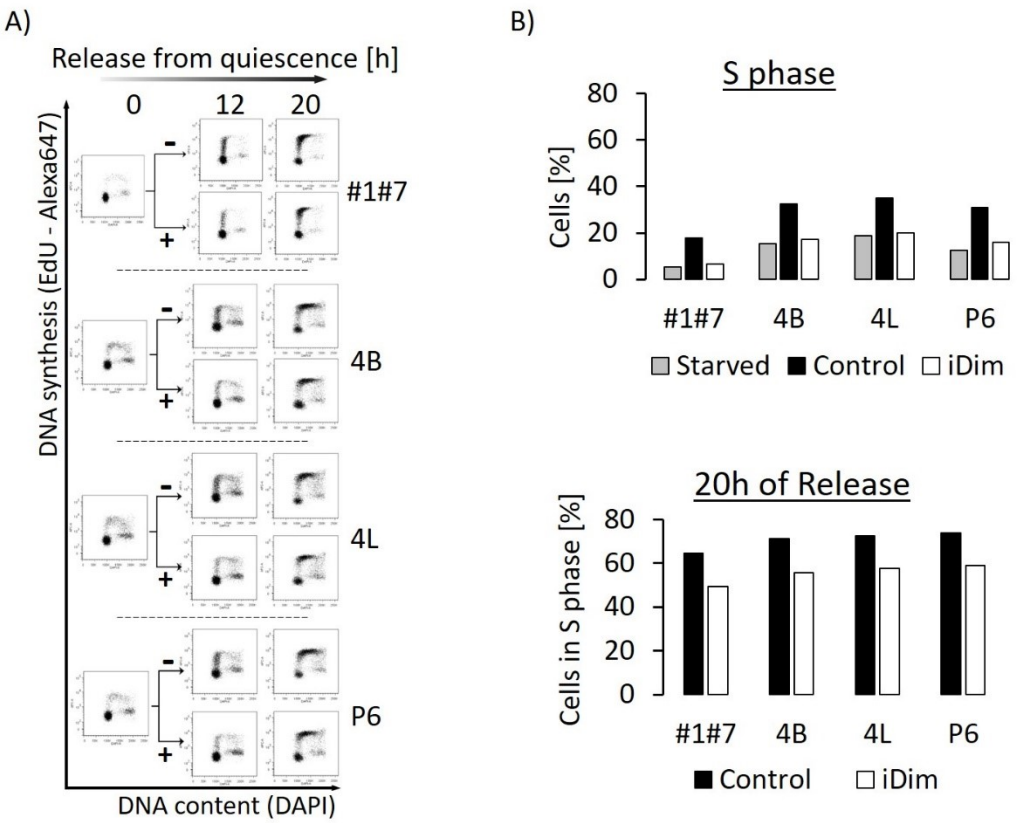

Figure 20. Behavioural comparison of twice-sorted cells with the parental T98G#1#7.

A) Comparison of serum-stimulated cell cycle re-entry of quiescent once-sorted with the double-sorted T98G#1#7 in absence (-) or presence (+) of 500nM heterodimerizer. Cells were rendered quiescent after 72 hours of serum withdrawal. Cell cycle distribution was achieved through EdU-pulse labelling/DAPI flow cytometry. B) Quantifications of S phase for starved cells, 12 and 20 hours of release after serum addition. Measurements were performed once for 4B, 4L and P6 populations and in triplicate for the T98G#1#7.

All tested clones (E9, G4 and G10) had a better arrest in G0G1 phase and subsequently a lower S phase entry (12h of release) and progression (20h of release) compared to the parental T98G#1#7 (Fig. 21). These results indicated that a more homogenous culture led to a better inhibition of Ras activity which in turn induced a higher reduction of S phase entry in serum-stimulated quiescent cells. For this reason, all further experiments were performed with the clones and will be referred as T98G#1#7.

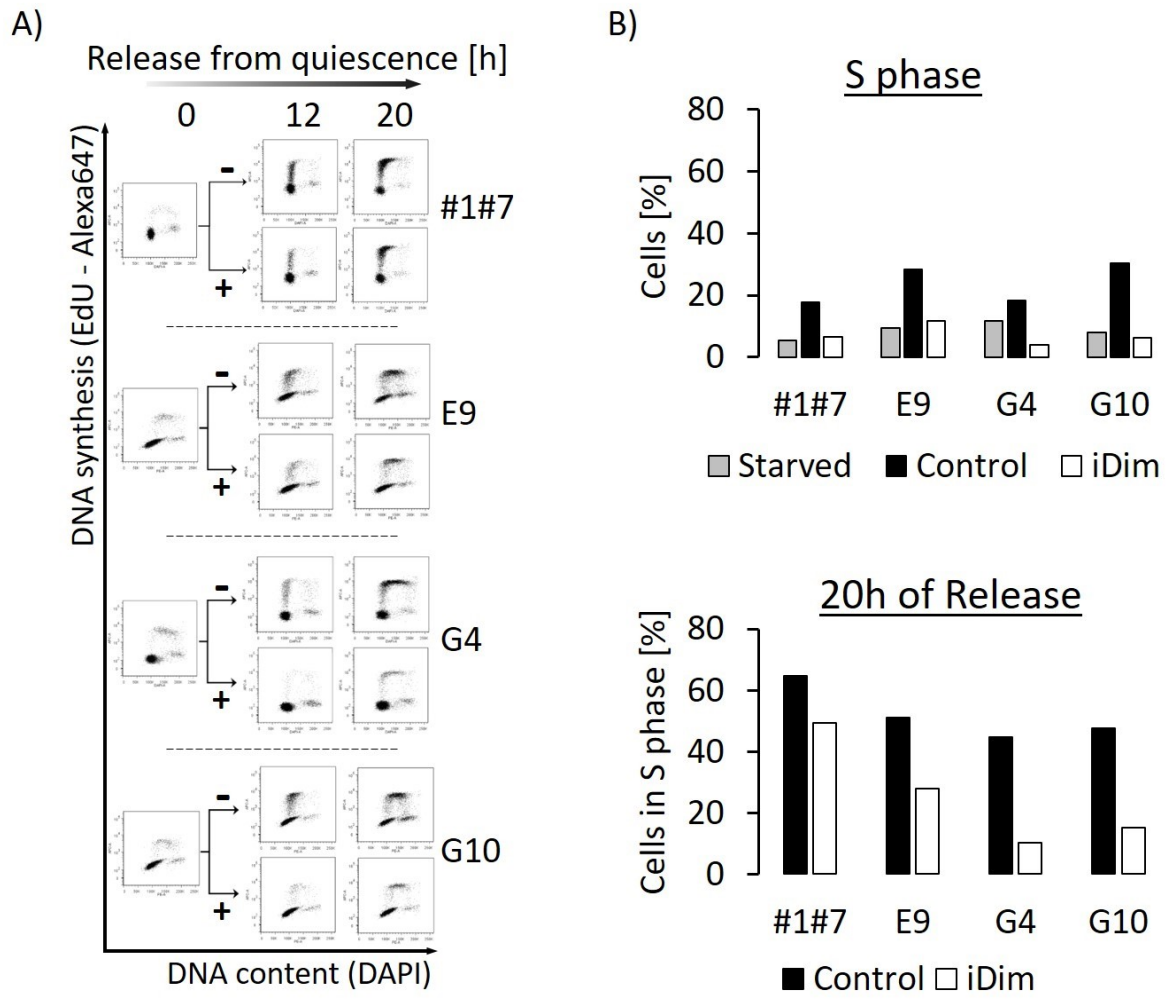

Figure 21. Behavioural comparison of monoclonal cultures with the parental T98G#1#7 cells.

T98G#1#7 cells were made quiescent through 3 days of serum withdrawal and subsequently stimulated to re-enter cell cycle in the absence (-) or presence (+) of 500nM heterodimerizer. Cell cycle re-entry and progression was followed up to 20 hours after serum stimulation. A) Cell cycle profiling was performed using the EdU-pulse labelling / DNA staining flow cytometry. B) Quantifications of S phase for starved cells, after 12 (upper graphic) and 20 hours of release (lower graphic).

4.4. Validating the specificity of the RasOFF system

4.4.1. Mutant NF1(R1276P) has no effect on Ras activity

A system to manipulate dynamic signalling cascades must be validated with appropriate controls. As shown earlier, HeLa #1#7 cells behaved just as wild type HeLa, unless treated with the heterodimerizer. This demonstrates that the presence of constructs #1 and #7 do not by themselves affect Ras activity or its downstream effectors in any detectable way.

Additionally, a second HeLa#1#7 cell line harbouring a mutant, inactive effector unit #7 EGFP-FRB-NF1(R1276P) shown to abrogate its RasGAP activity (Klose, Ahmadian *et al.* 1998) was generated as an independent control for the specificity of the heterodimerizer system. Transduced cells were puromycin-selected for 1 week and sorted for red/green double positive cells. The sorted cells will be referred to as HeLa#1#7NF1(R1276P). Expression of the two units was confirmed by western blot analysis (Fig. 22A). Wild type HeLa cells were used as a negative control, whereas HeLa cells expressing only the anchor #1 or effector unit #7NF1(R1276P) served as single positive controls.

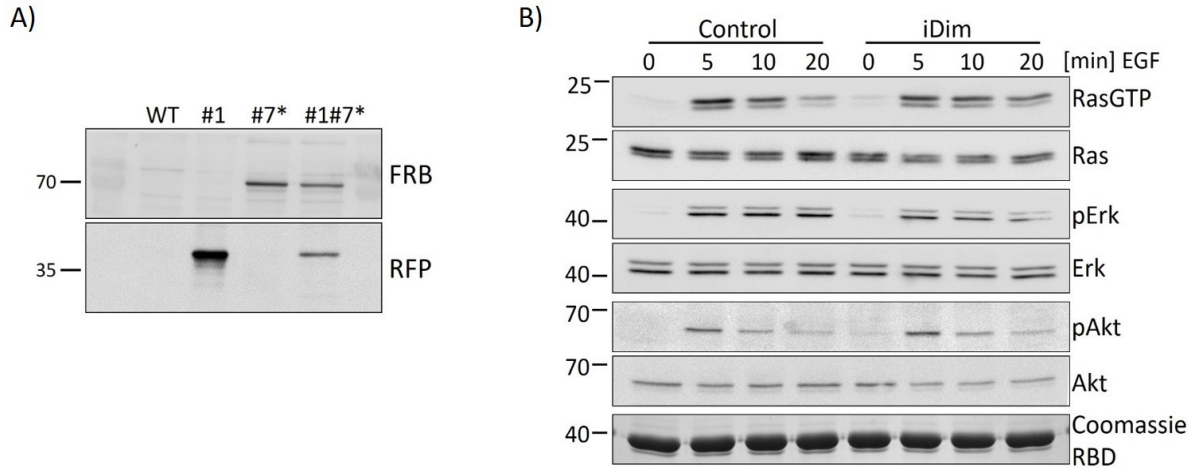

Figure 22. HeLa#1#7NF1(R1276P) have no alteration in Ras activity upon heterodimerizer administration.

A) Expression of the anchor #1FKBP-mCherry-K-Ras-hvr and effector unit #7EGFP-FRB-NF1(R1276P) in stably transduced HeLa cells. Wild type HeLa cells were used as a double negative control, whereas HeLa stably transduced with the anchor or effector unit served as single-positive controls. *mutant #7FRB-EGFP-NF1(R1276P) B) Representative immunoblot of HeLa#1#7NF1(R1276P) treated or untreated with heterodimerizer. Cells were starved overnight and incubated for 1 hour with 500nM heterodimerizer before being stimulated with 10ng/ml EGF at the indicated times points. Samples were further prepared for Ras-GTP pulldown assay. Coomassie staining served as a loading control.

To characterize these cells, Ras activity was verified in HeLa#1#7NF1(R1276P) upon EGF stimulation, which were previously starved and treated with heterodimerizer (Fig. 22B). As expected, no alterations in Ras activity or its downstream effector Erk was detected.

To exclude the possibility that for some unknown reason the induced translocation of the mutant effector unit toward the plasma membrane does not occur, confocal microscopy experiments were performed with HeLa#1#7NF1(R1276P). As shown in figure 23 the anchor

unit #1 is bound to the plasma membrane while the effector unit #7NF1(R1276P) is spread throughout the cytoplasm. The addition of the heterodimerizer resulted in the co-localization of the effector and anchor unit at the plasma membrane.

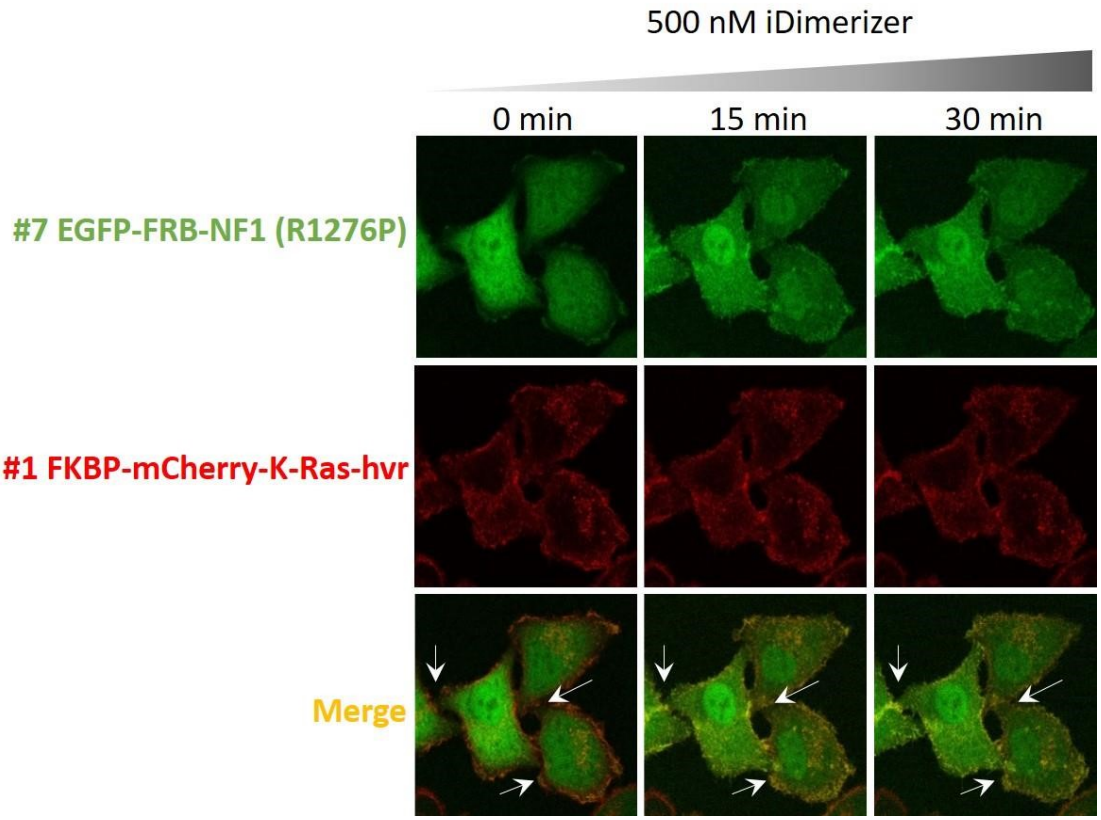

Figure 23. Heterodimerizer-induced translocation of the cytoplasmic unit #7NF1(R1276P) towards the membrane-bound unit #1FKBP-mCherry-K-Ras-hvr in HeLa cells.

Stably transduced HeLa#1#7NF1(R1276P) cells were incubated with 500nM heterodimerizer. Confocal microscopy images were taken prior and after heterodimerizer addition.

4.4.2. The heterodimerizer alone has no effect on wild type T98G cells

The heterodimerizer AP21967 used to activate the system is a rapamycin analogue that no longer has the ability to interact with the wild type form of FRB, part of the NF1-coupled effector unit #7. To verify that the heterodimerizer itself does not have any effect on Ras activity, a RasGTP pulldown assay was performed using wild type T98G cells (Fig. 24A). EGF stimulation of the control serum-starved T98G cells induced an immediate Ras activation, which was not altered by the administration of the heterodimerizer. Erk protein was also equally activated in untreated and treated T98G cells.

The lack of effect of the heterodimerizer on cell cycle progression was also investigated. Wild type T98G cells were first made quiescent and then stimulated with serum to re-enter the cell cycle in the absence or presence of the heterodimerizer. Since the heterodimerizer is dissolved in 100% ethanol, treatment of wild type T98G cells with the latter served as internal control. As expected, no difference was found in S phase (20h of release) between the control and the treated cells. To further confirm these results, T98G cells stably transduced only with the anchor unit #1 (designated as T98G#1) were made quiescent and re-stimulated to enter the cell cycle with and without heterodimerizer. S phase quantifications showed no significant difference between the control and treated T98G#1 cells. The results obtained are presented in figure 24 B and C.

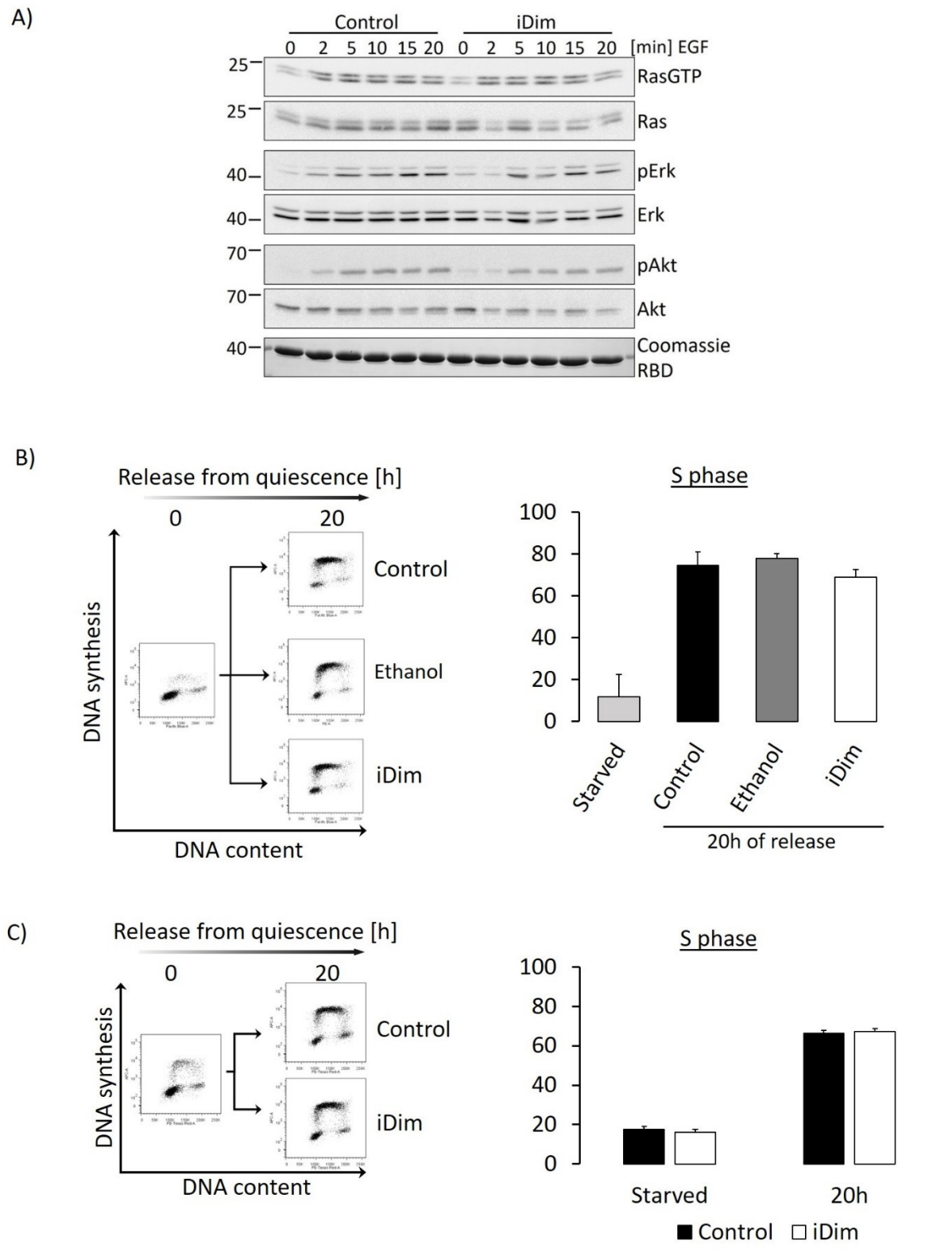

Figure 24. Heterodimerizer administration has no effect on wild type T98G cells emerging from quiescence.

A) Overnight starved T98G cells were treated or not with 500nM heterodimerizer for 1 hour and further spiked with 10ng/ml EGF for the indicated time points. RasGTP pulldown and western blotting was performed to detect Ras, Erk and Akt activity. Coomassie staining served as a loading control. B) T98G cells were made quiescent after 72 hours of serum withdrawal (0.15% FCS) and released in the cell cycle in the presence or absence of

500nM heterodimerizer or Ethanol. Cell cycle profiles were analysed by click chemistry-based fluorescent labelling and flow cytometry in combination with DNA content dye. Quantifications of S phase values are presented as mean ± SD of three independent experiments. C) T98G#1 cells were induce to quiescence after 72 hours of serum starvation (0.15% FCS) and further re-stimulated to re-enter the cell cycle with and without heterodimerizer (500nM). Quantifications of S phase values are presented as mean ± SD of two independent experiments.

4.5. Ras activity in G0/G1 and G1/S transition

4.5.1. Temporal profiling of Ras activity during G0-G1-S phase transition

The use of anti-Ras antibodies in cells emerging from quiescence demonstrated a requirement for Ras activity in multiple steps during G0-G1-S transition. Dobrowolski *et al.* identified the presence of 2 temporally distinct peaks of Ras activity during G1 phase: one in the beginning and one in mid-to-late G1 stage (Dobrowolski, Harter *et al.* 1994). In the absence of an expeditious approach to modulate Ras activity, its frequency and amplitude required during G1 phase is still unclear. Given these conditions, we started investigating the activity of Ras and its downstream effector kinases Erk and Akt in T98G#1#7 cells emerging from quiescence into G1. Cells were induced to acquire quiescence by serum withdrawal and then stimulated to re-initiate cell cycle with or without heterodimerizer treatment, either at release or 1h later. At the indicated time points, samples were prepared for a Ras-GTP pulldown assay followed by western blotting.

As a very first observation in this cell line, we noticed a lack of a genuine peak for Ras activity at mid/late G1 phase, but a rather continuous basal level until the end of time course studied. Growth factor stimulation induced an immediate Ras activity that reached its peak within the first 10 minutes. Upon heterodimerizer administration, Ras-GTP levels and its major downstream effectors (p-Erk) are strongly decreased. Phosphorylation levels of Akt remain unaltered, indicating its regulation is independent of Ras activity at this juncture in the cell cycle (Fig. 25).

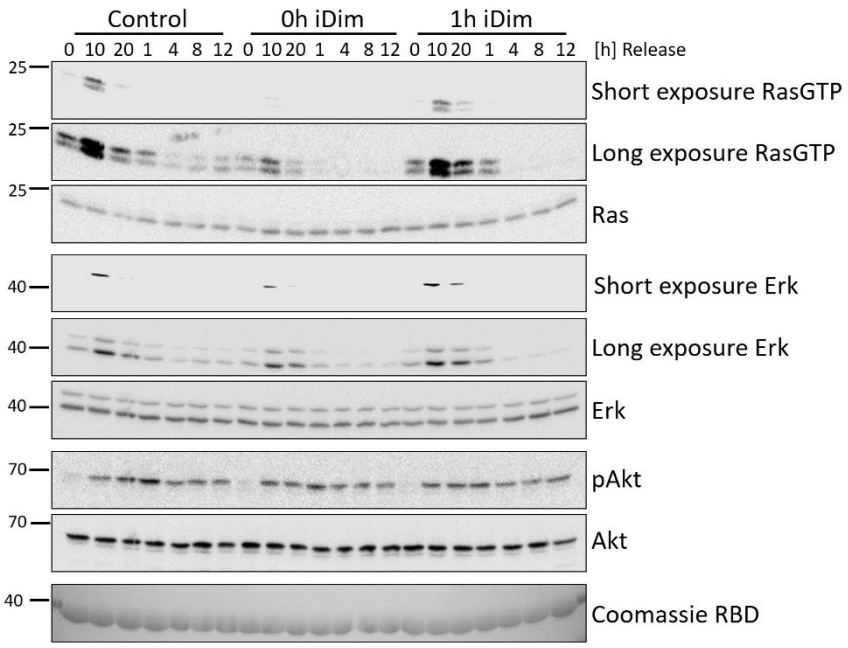

Figure 25. Kinetics of Ras activity in quiescent T98G#1#7 cells treated with heterodimerizer at release or 1 hour later.

After 72 hours of starvation with 0,15% FCS, T98G#1#7 cells were stimulated to re-enter cell cycle with growth media supplemented with 10ng/ml EGF and treated with heterodimerizer at release (0h iDim) or 1 hour later (1h iDim). At the indicated time points samples were processed for RasGTP pulldown assay and western blotting. Coomassie staining served as a loading control.

Under identical settings, experiments were performed to investigate cell cycle distribution of cells emerging from quiescence when Ras activity was turned off. T98G#1#7 deprived of Ras function were not able to enter the cell cycle which has been also shown in previous studies. Interestingly, blocking Ras after the first activity peak resulted in the same outcome, suggesting that Ras activity is necessary beyond that first activity peak for cells to progress into S-phase. Our results evidence the obligate requirement of Ras function in cells emerging from quiescence not only in early G1 but also later. Gille and Downward demonstrated that multiple signalling pathways were involved in mediating Ras-induced G1 phase progression of quiescent cells after serum stimulation (Gille and Downward 1999). Along with the MAPK pathway, the second main player was the activation of PI3kinase that induced cyclinD1 transcription and E2F activity through serine/threonine kinase Akt/PKB. To discriminate between the two most direct signalling cascades relayed from active Ras during G0 to G1 transition, quiescent T98G#1#7 cells were released in presence of two different inhibitors, U0126 and LY294002. The utility of U0126, a highly selective inhibitor of the MAPK pathway

at the level of MEK, was exploited as an alternate means of interfering with this pathway, in addition to Ras. LY294002 is a potent PI3K pathway inhibitor that inhibits cell cycle progression through the Akt-FOXO-CyclinD1 axis (Muise-Helmericks, Grimes *et al.* 1998).

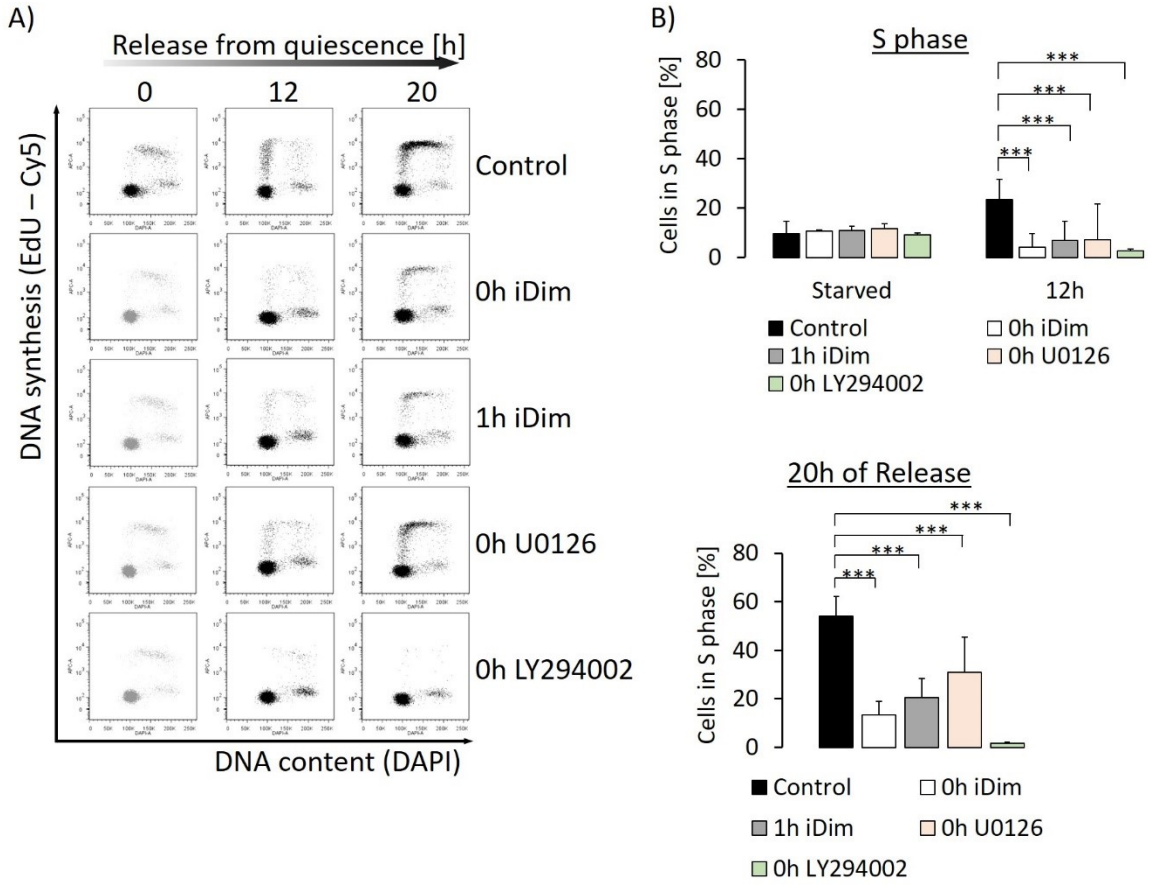

Figure 26. Cell cycle distribution of quiescent T98G#1#7 cells stimulated to re-enter the cell cycle in the presence of heterodimerizer, MEK or PI3K inhibitor.

T98G#1#7 cells were induced to exit the cell cycle through 72 hours of serum withdrawal (0.15% FCS). Re-entry and progression into the cell cycle were performed in absence or presence of 500nM heterodimerizer or one of the inhibitors: 10µM U0126 (MEK inhibitor) and 25µM LY294002 (PI3K inhibitor). A) Global nucleotide incorporation and cell cycle profiles were obtained using the EdU-pulse labelling / DAPI flow cytometry. Repetitions of the same time points are shown for reasons of clarity (grey). B) Quantification of S phase represented as mean ± SD of three independent experiments. Statistical significance was calculated using two-way ANOVA. ***p<0.001, 0h iDim/U0126/LY294002: treatment at release. 1h iDim: treatment 1 hour after release.

As shown in figure 26, PI3K inhibition abrogated cell cycle progression completely as expected, whereas MEK inhibition resulted in an effect similar to the heterodimerizer treatment, suggesting that MEK-Erk pathway is the likely mediator of Ras signals under these circumstances.

4.5.2. An alternative method to analyse cell cycle progression

Under an agreement with ChemoMetec GmbH, we had access to NucleoCounter® NC-3000™, a multicolour fluorescent cell-analysing instrument that uses an automated image cytometry technology to characterize cell properties. The software captures several images for each sample pre-stained with DAPI (a nuclear DNA content stain) and presents the results of the cell cycle distribution as histograms. Quiescent T98G#1#7 cells were triggered to start proliferating upon serum addition in the presence of the heterodimerizer or MEK/PI3K inhibitors. After 24 hours, cells were harvested and prepared for analysis with the NucleoCounter® NC-3000™. Also in this case, inhibition of Ras activity in cells emerging from quiescence lead to an arrest in G0 regardless of the time of the administration of the heterodimerizer to the cells. The PI3K inhibitor, LY294002, strongly blocked G1 phase entry and progression, while the MEK inhibitor, U0126, induced cell cycle arrest of cells to the same extent as the heterodimerizer (Fig. 27).

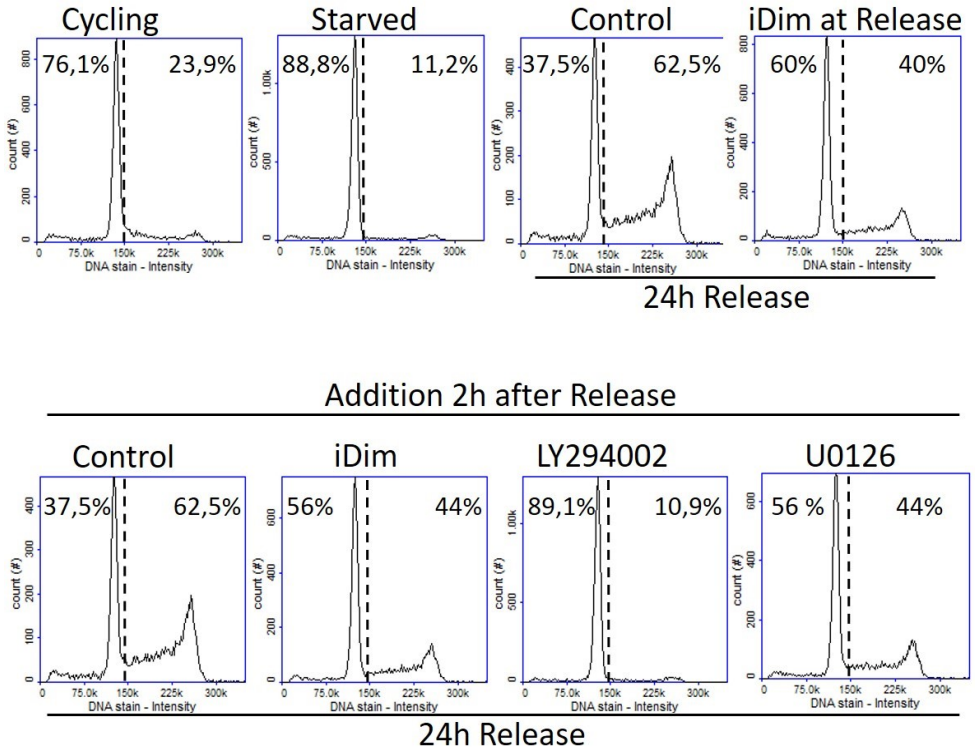

Figure 27. Fluorocytometric analysis confirms a block in cell cycle progression in cells treated with heterodimerizer (iDim).

T89G#1#7 cells were rendered quiescent after 3 days of serum starvation and released back into the cell cycle in the presence or absence of the indicated drugs. The heterodimerizer and inhibitors were administered simultaneously with release or 2 hours later, as labelled. Cell cycle profiling was performed by DAPI staining and

cytophotometry analysis using the NucleoCounter® NC-3000™ device 24 hours after release. Dotted line is an arbitrary gate separating G0/G1 from S-G2-M phases. The percentages on each histogram show the quantifications of G0G1 versus S-G2-M phases. UO126: MEK inhibitor. LY294002: PI3K inhibitor. 0h iDim: treatment at release. 2h iDim/U0126/LY294002: treatment 2 hours after release.

4.6. At which time point does the G1 phase become independent of Ras signalling?

The mandatory requirement of Ras during G1 phase of cells emerging from quiescence has been proved by many studies, however the duration of this dependency is not yet clear. To determine whether Ras activity is continuously required and indispensable to G1 phase progression, we continued our investigation by turning off Ras, advancing periodically, throughout G1 phase in cells released from quiescence. Previous experiments indicated that quiescent T98G#1#7 cells need between 10 and 12 hours after serum stimulation to transit through G1 and begin S phase. Therefore, samples were treated with heterodimerizer such that Ras was switched off every 1 hour throughout G1 and were followed until 20 hours after being released. Figure 28 consolidates cell cycle distribution of the entire series of samples at 12 and 20 hours of release.

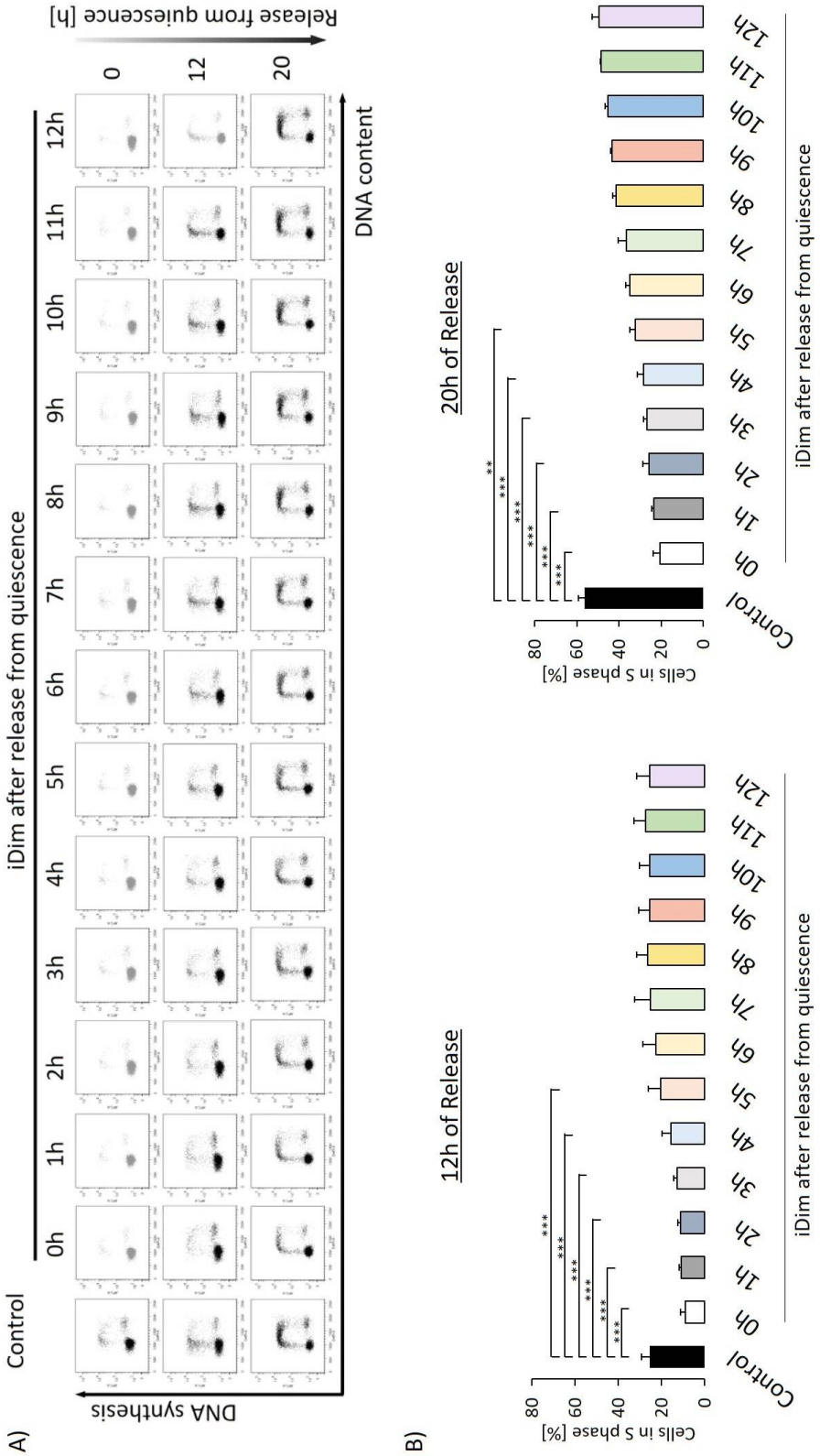

Figure 28. Administration of the heterodimerizer throughout G1 phase for T98G#1#7 cells emerging from quiescence.

T98G#1#7 cells were made quiescent after 72 hours of serum deprivation (0.15%FCS). Mitogenic –induced cell cycle re-entry and progression was tracked up to 20 hours. A) Cell cycle profiling of T98G#1#7 cells emerging from quiescence. Cell cycle distribution was obtained by EdU-incorporation/DAPI flow cytometry. Treatment with the heterodimerizer was perform every 1 hour throughout G1 phase. Repetitions of the same time point are shown for reasons of clarity (grey). B) Quantification of S phase after 12 and 20 hours of release. Values are presented as mean ± SD of three independent experiments. Statistical significance was calculated using two-way ANOVA. **p<0.01, ***p<0.001

Administration of the heterodimerizer up to 5 hours after serum stimulation, significantly reduced G1 to S transition and progression. Inhibition of Ras activity later during G1 phase had no effect on S phase entry or progression. As previously shown, T98G#1#7 were characterized by only one peak in Ras activity in early G1, which is not enough to drive cells through G1 and S phase. These results indicate that cells emerging from quiescence depend on mitogenic Ras signals at least until mid G1 phase.

Cell cycle progression is controlled by a specific and well conserved mechanism composed by several cyclin-cyclin dependent kinases (CDKs) complexes specific for every phase of the cell cycle. Progression through G1 phase and commitment to S phase is supported by cyclinD-Ckd4/6 complexes (Bartek and Lukas 2001). Injection of anti-cyclinD1 and anti-Ras antibodies in NIH3T3 cells emerging from quiescence blocked entry into S phase at the same time, approximately 4 hours earlier (Hitomi and Stacey 1999b), proving the simultaneous requirement of Ras and cyclinD1 in G1 to S phase transition. On the other hand, Gille *et al.* showed that Ras signalling induces cyclinD1 expression (Gille and Downward 1999). To confirm this possibility in our system, quiescent T98G#1#7 were stimulated with serum to re-enter the cell cycle under different schedules of turning Ras off: 1) released in total absence of Ras function (0h iDim); 2) blocking Ras activity after cells experienced the early Ras activation (1h iDim) and 3) blocking Ras in mid-G1 phase when its function is less required (6h iDim) (Fig. 29).

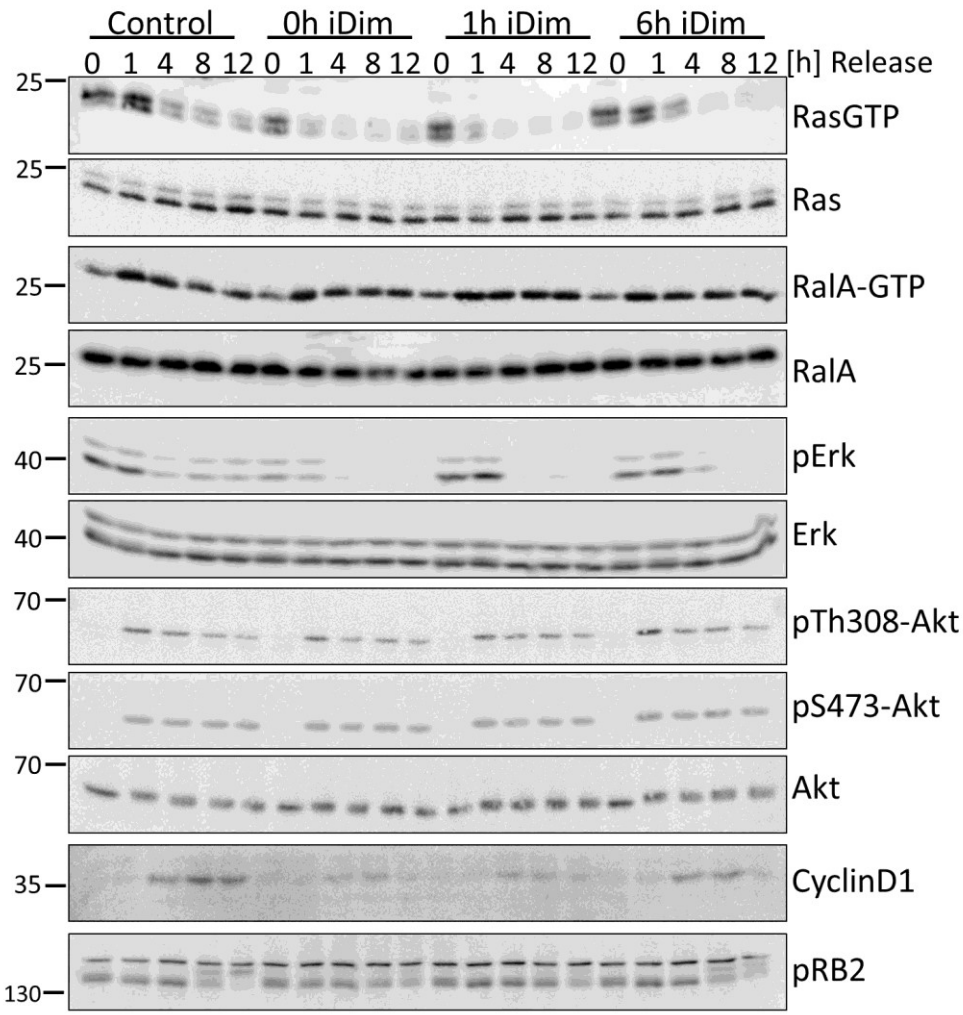

Figure 29. G1 phase expression of cyclinD1 is dependent on Ras activity in cells emerging from quiescence.
Representative immunoblot (1 of n=3) of downstream effectors of Ras upon heterodimerizer administration at release and 1 or 6 hours later. Quiescent T98G#1#7 cells were stimulated with serum supplemented with 10ng/ml EGF to re-enter and progress through G1 phase in absence or presence of 500nM heterodimerizer. At the indicated time points cells were lysed and prepared for western blotting.

Treatment of T98G#1#7 cells with heterodimerizer declined Erk activity, while the small G protein RalA and Akt function remained undisturbed. In the control set, cyclinD1 activity was detectable only after 4 hours of release. Switching off Ras before or shortly after serum stimulation of quiescent cells induced a significant downregulation in cyclinD1 expression levels. Delaying Ras inhibition only modestly decreased cyclinD1 levels; these CyclinD1 levels were apparently sufficient to drive cells into S phase. These differences in the expression of cyclinD1 are confirmed by the change in phosphorylation levels of retinoblastoma protein (pRB2). In quiescent cells, pRB is active (hypophosphorylated state) and bound to E2F

transcription factor blocking entry into S phase. pRB inactivation (hyperphosphorylated state) by cyclinD1-CDK4/6 complexes results in the release of E2F that in turn promotes expression of genes necessary for S phase progression (Giacinti and Giordano 2006). The shift between hypo- and hyper- phosphorylation levels of pRB2 is visible in the control and the 6h iDim set where cyclinD1 expression is high and cells progress through G1 and enter S phase. The opposite was observed in cells lacking Ras activity and as a result of lower cyclinD1 expression.

4.7. Investigating the role of Ras in cycling T98G#1#7 cells

4.7.1. Asynchronous cycling cells arrest in G0G1 in absence of Ras activity

Literary evidence claims that in actively proliferating cells, the commitment to proliferate is not made during G1, but rather in the preceding G2 phase in a mitogen-induced Ras-dependent manner (Hitomi and Stacey 1999b). Following this argument, we started treating asynchronous cycling T98G#1#7 cells with heterodimerizer and then analyse their distribution throughout the cell cycle. Cells were cultured in the presence of the heterodimerizer for 48 hours and the results are shown in figure 30. The effect of the treatment is already visible at 24 hours and is enhanced at 48 hours. Switching off Ras protein forced cells to arrest in G0G1 phase at the expense of S and G2M phase.

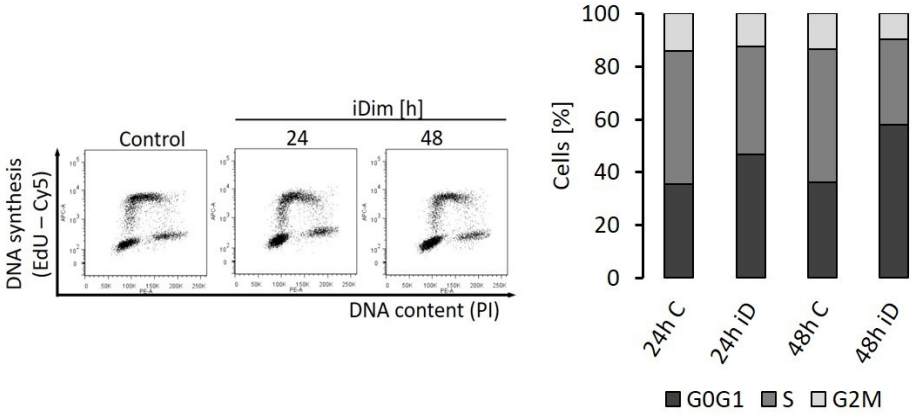

Figure 30. Activation of RasOFF system induced time-dependent accumulation in G0G1 phase of actively proliferating cells.

Actively cycling cells were kept in the absence or presence of 500nM heterodimerizer for 48 hours. Cell cycle profiling was obtained through EdU-pulse labelling/PI flow cytometry (on the right). Quantifications of the cell cycle distribution shown as percentage of total (on the left).

4.7.2. Synchronized T98G#1#7 cells

In order to investigate whether Ras dictates commitment to subsequent proliferation in the current G2 phase, two different strategies were tried in parallel. The first one synchronizes the cells in G1/S border through thymidine treatment, while in the second strategy cells are arrested in G2 phase by a double block with thymidine and RO3306. In both cases T98G#1#9 were followed until the next S phase and flow cytometry for cell cycle distribution was performed. In both cases, T98G#1#7 cells released from the G1/S or G2/M block after removal of the inhibitor continued progression in the next step of the cell cycle as one single population, simplifying the process of following cell transition between phases.

In cells released from the G1/S block, Ras was turned off at three different moments: at the beginning of the release (0h iDim set), in early-mid S phase (3h iDim set) and mid-late S phase (6h iDim set) (Fig. 31). Despite the treatment, cell cycle progression from S to G2M phase and G2M to G0G1 phase was independent of Ras activity. Whereas entry in the next S phase was significantly reduced and cells accumulated in G0G1 phase.

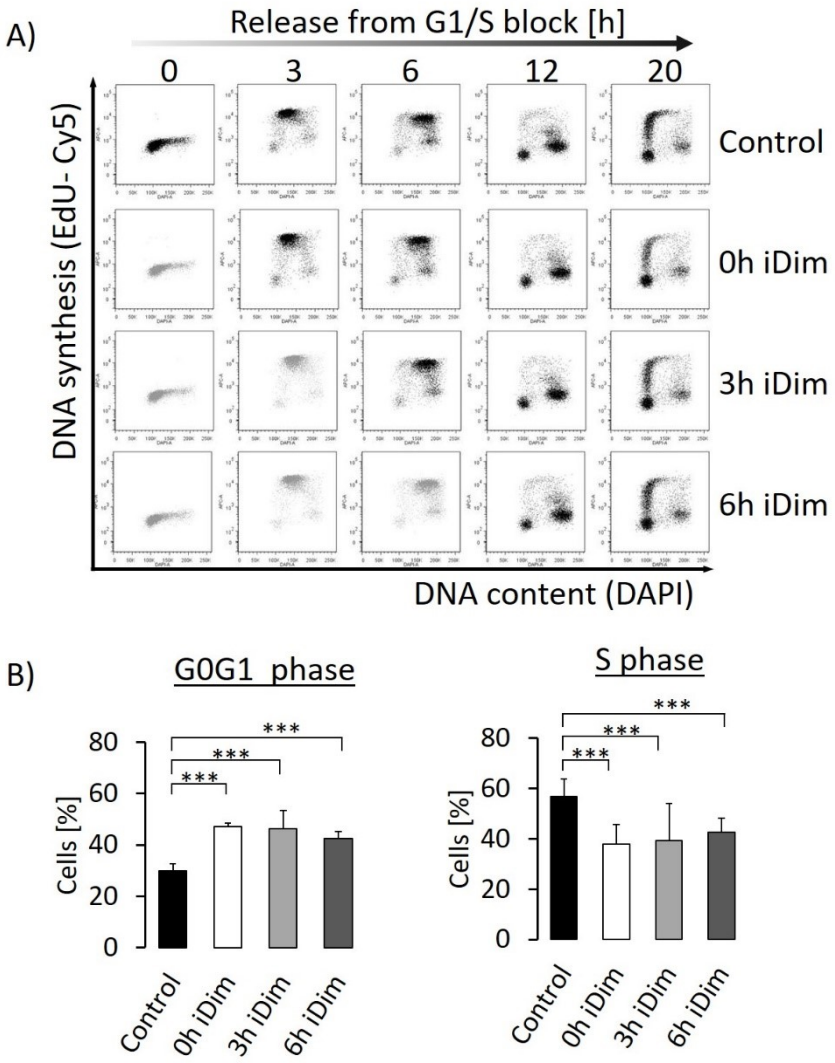

Figure 31. T98G#1#7 cells released from G1/S block are blocked in the next G0G1 phase in absence of Ras activity.

Cells were synchronized at the G1/S border after 24 hours of treatment with 2mM thymidine. Release into the cell cycle was performed in absence or presence of 500nM heterodimerizer at release or later. A) Representative time course of cells released from G1/S block. Cell cycle distribution was achieved through EdU incorporation/DAPI flow cytometry. Time point repetitions are shown for reasons of clarity (grey). B) Quantifications of G0G1 and S phase at 20 hours after release. Values are shown as mean ± SD of three independent experiments. Statistical significance was calculated using two-way ANOVA. ***p<0.001, 0h iDim: heterodimerizer administration at release. 3h iDim or 6h iDim: heterodimerizer administration 3 or 6 hours after release.

With the second approach, when cells were synchronized in G2, Ras activity was blocked from the beginning of the release or every 2 hours from G2/M to G1/S transition (Fig. 32). Also in this case, progression of the cells in the next G1 phase was independent from Ras activity. In

contrast, S phase entry was significantly lower when Ras was turned off before mitosis (0h iDim set) and up to 4 hours after being released from the block (2h, 4h iDim sets).

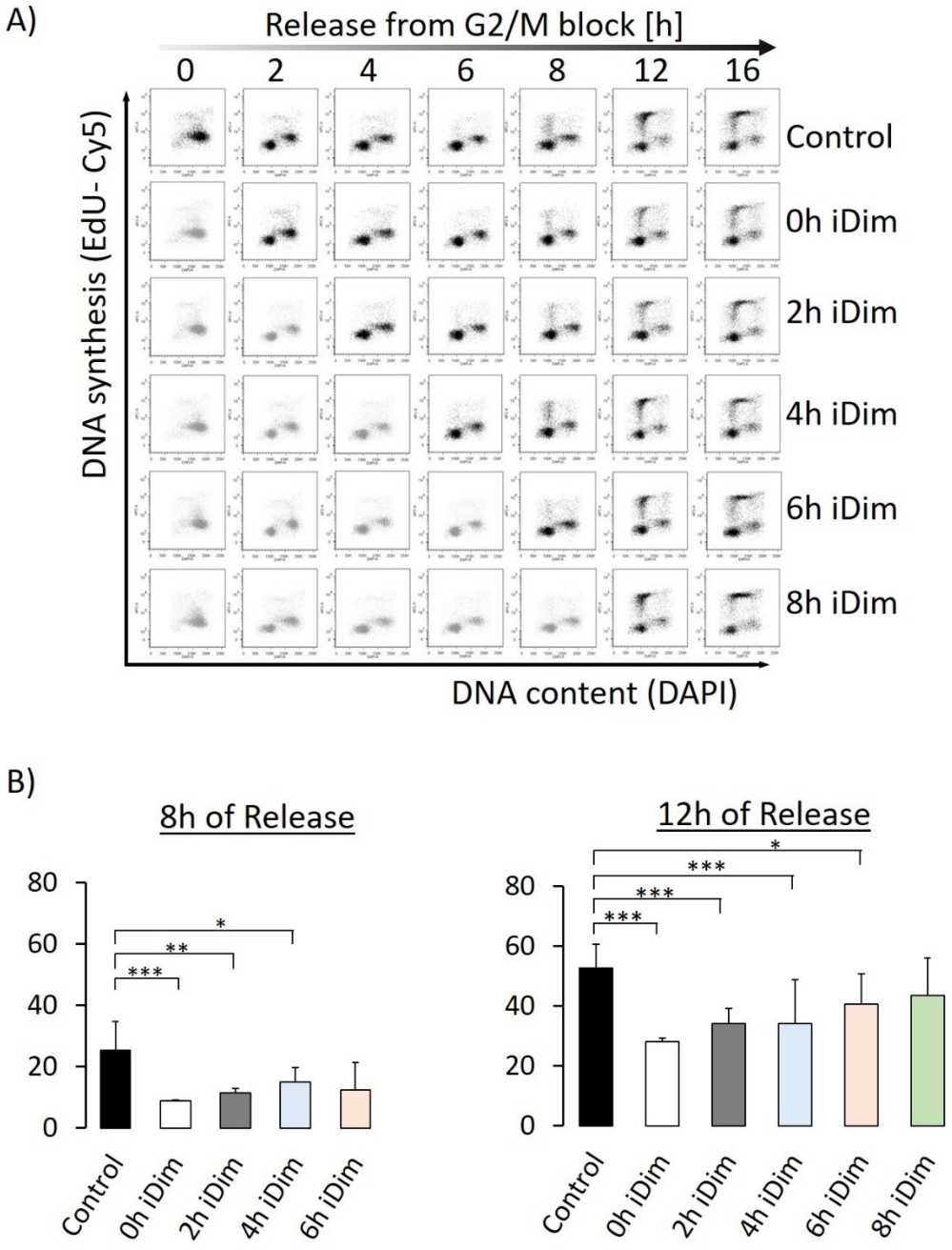

Figure 32. Release of T98G#1#7 cells from G2/M block in absence or presence of the heterodimerizer.

Synchrony at G2/M border was achieved by a double block with thymidine/RO3306. Removal of inhibitors induced release of the cells into the cycle. The heterodimerizer (500nM) was administrated at release (0h iDim) or 2, 4, 6 and 8 hours after release (2h iDim, 4h iDim, 6h iDim and 8h iDim, respectively). A) Representative determination of the cell cycle progression upon release through EdU labelling/DAPI flow cytometry. Time point repetitions are shown for reasons of clarity (grey). B) Quantifications of early (8 hours) and early/middle (12

hours) S phase. Values are presented as mean ± SD of three independent experiments. Statistical significance was calculated using two-way ANOVA. *p<0.05, **p<0.01, ***p<0.001

To further confirm these results, the expression and activity of two cell cycle regulators, cyclinD1 and pRB, was investigated for cells released from the G2 block in the absence or presence of the heterodimerizer, either at release or 6 hours later. Upon heterodimerizer treatment, Erk phosphorylation is downregulated in both treated sets compared to the control, while Akt activity remains unchanged. CyclinD1 levels are detectable at the end of the synchronization, since cells are blocked in G2 phase by the double synchronization method, in line with previous studies (Yang, Hitomi *et al.* 2006). Turning off Ras before T98G#1#7 are released from the G2 block induced a decline in cyclinD1 levels, but did not block it completely. Retinoblastoma phosphorylation levels (pRB2) are detectable only 4 hours after release because at that time cells are in mid G1. The fluctuation between the hypo- and hyper- phosphorylated form reflects the changes in cyclinD1 expression (Fig 33). Ras–induced downregulation of cyclinD1 leads to accumulation of pRB2 in the hypophosphorylated form.

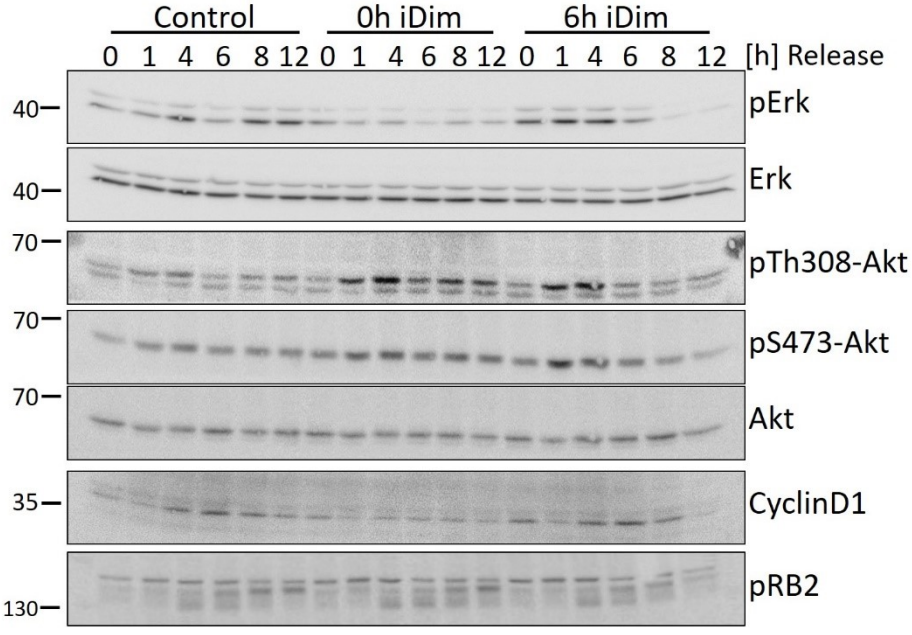

Figure 33. CyclinD1 expression in T98G#1#7 cells released from G2/M block in the presence of the heterodimerizer.

Representative immunoblot (1 of n=3) of Ras downstream effectors. T98G#1#7 were synchronized at G2/M border through a double block with thymidine/RO3306 and further released into the cell cycle upon inhibitors

removal. Administration of the heterodimerizer (500nM) was done at release (0h iDim) or 6 hours after release (6h iDim). At the indicated time points cells were lysed in RIPA buffer and processed for western blotting.

4.8. Investigating the role of Ras during G2 phase in cycling HeLa#1#7 cells

The correlation between Ras activity and cyclinD1 in G1 was also detected in HeLa#1#7 cells. By using a double block of thymidine and nocodazole, HeLa#1#7 were synchronized in mitosis and then released into the cell cycle. Ras activity was turned off 3 hours after release, in which more than 50% of the cells are entering G1. Untreated HeLa#1#7 cells expressed two distinct peaks of Ras activity, one in early G1 (1h after release) and a second time towards the end of G1 phase (10 hours after release) (Fig.34). Surprisingly, we observed a band shift of phosphorylated Akt from residue Threonine308 in the early phase of release to Serine473 towards the end of our experimental time points. The addition of the heterodimerizer downregulates Ras-GTP levels reflected in the decline in Erk activity. No alteration of Akt phosphorylation level is detected. CyclinD1 expression reached a peak toward the end of the G1 phase in the control and also showed that there was no difference in the treated cells.

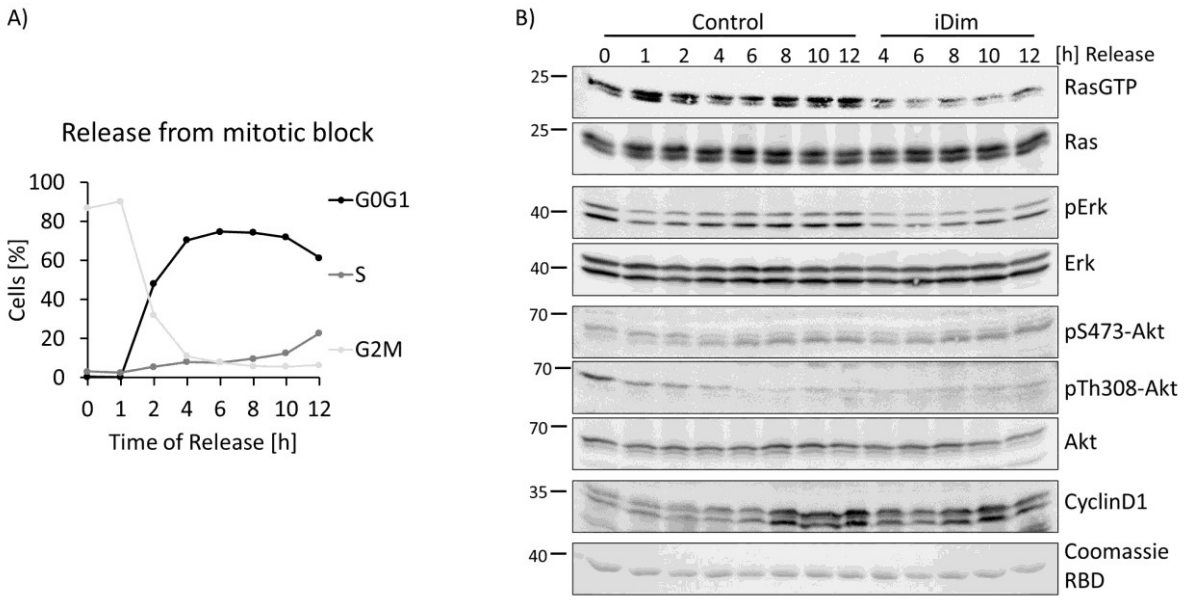

Figure 34. Ras kinetics of HeLa#1#7 cells released from mitotic block.

HeLa#1#7 cells were synchronised in mitosis through a double block with thymidine/nocodazole and subsequently released into the cell cycle in absence or presence of 500nM heterodimerizer. The release medium was supplemented with 10ng/ml EGF. At the indicated time points cells were prepared for flow cytometry or western blot analysis. A) Tracking the cell cycle progression of mitotic HeLa#1#7 was monitored through propidium iodide FACS analysis for DNA content. B) Representative immunoblot (1 of n=3) of heterodimerzer-

induced effect on Ras activity and its downstream effectors. The heterodimerizer was administrated 3 hours after cells were released. Coomassie staining served as a loading control.

Furthermore, it has been reported that cyclinD1 can be regulated by the PI3K/Akt pathway through FOXO (Schmidt, Fernandez de Mattos *et al.* 2002). To evaluate PI3K-dependent cyclinD1 expression, in parallel with the heterodimerizer, cells were treated with the PI3K inhibitor, LY294002. HeLa#1#7 were subjected to the same experimental conditions as previously described. In addition, a highly selective inhibitor of the MAPK pathway at the level of MEK, U0126, was exploited as an alternate means of interfering with MAPK pathway, downstream of Ras (Fig.35). Upon heterodimerizer treatment, Ras and subsequently Erk downregulation did not alter cyclinD1 expression. MEK inhibitor activated the negative feedback on Ras activity (Hennig, Markwart *et al.* 2016), but no alteration on cyclinD1 was observed. Surprisingly, under these conditions LY294002 did not block Akt activity and as result neither cyclinD1.

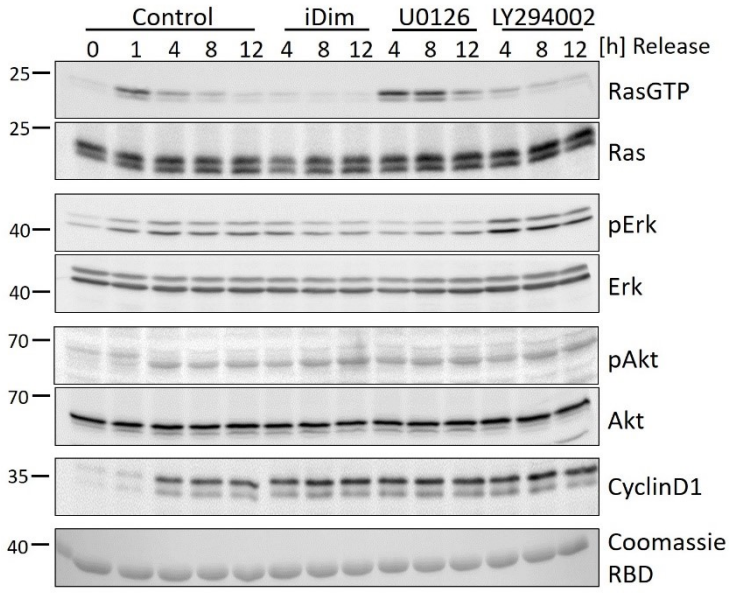

Figure 35. CyclinD1 expression is independent of Ras activity at midG1 in cycling Hela#1#7 cells.
Synchrony in mitosis was achieved through a double block with thymidine/nocodazole. Upon inhibitor removal, HeLa#1#7 cells were released into the cell cycle. After 3 hours of release they were treated with 500nM heterodimerizer, 10µM U0126 (MEK inhibitor) or 25µM LY294002 (PI3K inhibitor). At the indicated time points cells were prepared for Ras pulldown assay and western blotting. The image is a representative immunoblot out of three independent experiments. Coomassie staining served as a loading control.

5. Discussion

Ras is a small GTP-binding protein that exerts its function when associated with the plasma membrane. Ras protein has the ability to continuously cycle between an active GTP-bound and inactive GDP-bound state, under the supervision of two groups of regulators, GEFs and GAPs, respectively. Being identified as a key regulator of several signalling pathways like proliferation, differentiation, survival and morphology has placed Ras in the centre of the signalling transduction research for the last 30 years (Malumbres and Barbacid 2003). Nowadays, Ras is a well-known proto-oncogene and gain of function mutations are found in up to 30% of all human cancers with the highest incidence in pancreatic, colon and lung carcinomas (Bos 1989). Even though a lot of effort has been put in understanding Ras activation and interactions with up- and downstream proteins, the temporal dimension of Ras signalling is still not fully comprehended.

Ras has been qualified as one of the key players in the regulatory cell cycle machinery since it controls the re-entry of quiescent cells into the cell cycle and decides the cell's fate during G2 phase. Due to the lack of appropriate techniques, it is not yet completely clear at which precise point of the cell cycle Ras function is required or not.

Through this PhD thesis we will introduce a new experimental technique able to directly and accurately target Ras protein. It is based on the rapamycin-induced heterodimerization system and can manipulate Ras activity within minutes by switching it off. The heterodimerization system was used to interfere with Ras function at several moments during the cell cycle progression with the intention to better understand its effect during cell proliferation.

5.1 Characterization of the inducible heterodimerization system

5.1.1 Rapid, induced translocation of the cytosolic effector of the RasOFF system

The RasOFF heterodimerization system comprises one FKBP-coupled membrane associated unit (anchor unit) and one FRB-coupled cytoplasmic unit (effector unit). The anchorage of the

FKBP-coupled unit to the plasma membrane is accomplished by the K-Ras hvr CAAX sequence (Parker and Mattos 2015). The FRB-coupled unit was associated to the RasGAP, NF1. The inducible translocation of the latter toward the membrane-associated FKBP-coupled unit occurred in a short period of time and was visible after only 15 minutes. The speed of the translocation is directly dependent on the potential of the chemical dimerizer to immediately diffuse through the plasma membrane (Putyrski and Schultz 2012) and probably on the size of the protein coupled to the effector unit. The fast translocation and coupling of the two units emphasizes the greatest advantage of this system over others that exert their function in the range of hours to days. Majority of the contemporary experimental methods to manipulate Ras aim to alter the number of the proteins. They are transfection-based techniques that make use of plasmid DNA or interference RNA to either upregulate or repress Ras expression. The time required for the newly introduced RNA or DNA to be expressed within the cell is in the range of a few hours to days after transfection of the target cells. Apart from the time-consuming aspect of this method, the effect is often incomplete, non-specific, off-target and results are not always equally reproducible (Sledz and Williams 2005). These problems are encountered in every experiment performed. Another technique used to target Ras is the microinjection of neutralizing anti-Ras antibodies in single cells. The microinjection can be performed within 10 minutes in many types of cells, including adherent primary and dividing cell lines or suspension cultures, previously seeded on a coverslip. However, the disadvantage of this technique is the time required (between 2 hours and 1-2 days) for the preparatory and preliminary examinations before the microinjection. Many aspects must be taken into account during microinjection. Most of them are cell type specific and include the volume of the injected substance and the following incubation time to avoid the diluting effect, the injection pressure used during the procedure, number of microinjected cells and the survival rate after microinjection (Zhang 2007). In contrast, our heterodimerization system exerts its function within 15 minutes and the results are easily reproducible.

5.1.2. Acute inhibition of Ras activity

The physical translocation of the effector unit leads to the recruitment of the RasGAP, NF1, close to the plasma membrane where endogenous Ras in localized. The end result of this proximity showed an inactivation of Ras protein probably by accelerating the GTPase

hydrolysis. Decline in Ras activity was immediately reflected in its direct downstream effector, Erk. Interestingly, growth factor stimulation induced a documented transient Ras activation (Waters, Holt *et al.* 1995, Hennig, Markwart *et al.* 2016), while Erk activity was maintained for a longer period of time indicating a distinct inactivation mechanism. PI3K-Akt axis is activated upon EGF-stimulation of Ras and the transient EGF-induced Akt activation (Kumar, Afeyan *et al.* 2007) was downregulated together with Ras. This mechanism was not repeated in T98G cells, suggesting a cell type dependency.

Wild type HeLa cells were used as a control for the behaviour of HeLa#1#7 in the absence of the heterodimerizer. EGF and FCS stimulation of both cell lines presented a similar pattern of Ras activation and its effectors. In case of insulin-stimulation alone, a potent activator of PI3K pathway, Akt phosphorylation levels were higher. Under these circumstances, downregulation of Ras had no effect on Akt activity (data not shown).

HeLa#1#7 cells displayed a marginally lower Ras activity in general, compared to the wild type, parental HeLa cells, which might be due to the overexpression of the RasGAP–NF1, in the cytosol favouring higher basal rates of GTP hydrolysis. Another plausible explanation is the reduced EGFR and total Ras expression in HeLa#1#7 cells. Despite the differences in the amplitude of Ras activity, both wild type HeLa and HeLa#1#7 maintained equally proficient signalling downstream of Ras. Intriguingly, the overall expression of EGF receptors in HeLa#1#7 although lower than in wild type HeLa, receptor activation upon EGF stimulation was virtually unaltered in untreated and heterodimerizer-treated cells. Moreover, administration of the heterodimerizer did not alter the activity of two related small G proteins RalA and Rap1. Despite the shared characteristics of switch I and II regions among small G proteins (Caron 2003), the inhibitory effect of neurofibromin (NF1) did not affect RalA and Rap1 under these conditions. Simultaneously, the investigations on RalA activity, as the third downstream effector of Ras, demonstrated that Ras had no control on its function. Altogether our results strongly indicate that downregulation of Ras function upon heterodimerizer administration, is an effect of the FRB-mediated translocation of NF1 to the proximity of endogenous Ras. The results also prove that the RasOFF system exerts its function in a rather specific manner by inhibiting only Ras protein.

5.1.3. Dimerization of functional components alone constitute the RasOFF system

We have demonstrated that the RasOFF system is effective only when both fusion proteins and the heterodimerizer are available and fully functional. Our observations showed that the missense mutation R1276P in the NF1 protein completely abolished the inhibitory effect of the system on Ras protein, even though physical recruitment of the effector unit to the plasma membrane was not affected. Mutation of the arginine residue at 1276 into proline, as described in the literature, can completely disrupt the GAP activity of the arginine finger of NF1, without influencing NF1's secondary or tertiary structure and especially its ability to bind Ras GAP domain (Klose, Ahmadian *et al.* 1998). Several studies reported that neurofibromin inactivation, either through physiological proteosomal degradation or loss of function mutations, induced a higher amplitude and a longer duration of Ras activity in response to growth factors (Tan, Zhao *et al.* 2011, Hollstein and Cichowski 2013, Ratner and Miller 2015). In addition, in our experiments the use of mutant NF1 fusion protein led to a prolonged Ras activity, probably due to competition between endogenous and transduced NF1 molecules for the same Ras proteins.

The chemical inducer used to induce heterodimerization of the FKBP–FRB fusion proteins is a rapamycin analogue developed from Adria technologies and lacks the ability to interact with wild type FRB (Inoue, Heo *et al.* 2005). In addition, the FRB domain itself has a T2098L mutation that improves the affinity for inert analogues of rapamycin, but also impairs protein stability. Nonetheless, heterodimerizer-induced FBKP binding stabilizes the mutant FRB (Stankunas, Bayle *et al.* 2007). This feature of high affinity of the inert rapalog for mutant FRB, explains our results in wild type T98G and T98G#1 cells, where cell cycle progression and Ras activity were unperturbed in the presence of heterodimerizer, thereby suggesting a lack of interaction between the rapamycin analogue and wild type FRB.

5.2 Temporal dynamics of Ras activity during cell cycle progression

Seminal investigations on cellular proliferative fate decisions, to either continue dividing or embrace quiescence, spotted G1 phase as a pivotal point (Zetterberg, Larsson *et al.* 1995). Later studies in actively proliferating cells shed light on the G2 phase and mitogen-dependent commitment to either commit or refrain from the next cell cycle (Spencer, Cappell *et al.* 2013). In sum, these studies suggest that transition from quiescence (G0) to G1 phase is controlled

by the restriction point in G1, while for continuously cycling cells the commitment to complete another cycle is taken in G2 (Matson and Cook 2017). Regardless of the timing of the proliferation decision, mitogen-induced Ras activity is of central relevance (Mulcahy, Smith *et al.* 1985).

5.2.1. Advantages and disadvantages of the synchronization techniques

Throughout this project, we employed various cell cycle synchronisation techniques: 1. serum deprivation for 72 hours to obtain quiescent cells and 2. treatment with pharmacological agents alone or in combination such as thymidine, double block with thymidine - RO3306 and double block with thymidine - nocodazole, to arrest cells in G1/S border, G2 phase or mitosis, respectively. These pharmacological agents arrest cell cycle by specific mechanisms. Thymidine synchronises cells in G1/S border by inhibiting the activity of ribonucleotide reductase required for DNA synthesis (Reichard and Ehrenberg 1983). Nocodazole is a potent spindle poison and inhibits microtubule polymerization to arrest cells in mitosis (Merrill 1998). RO3306 induces G2 phase synchrony by binding to Cdk1 and interfering with the Cdk1-cyclinB complex formation required for G2-M transition (Vassilev, Tovar *et al.* 2006). The use of synchronising agents offered an important advantage especially in the investigation of cell-cycle phase-associated regulatory mechanisms. However, there is evidence suggesting some degree of undesirable or side effects caused by these drugs like alterations in the control of cell cycle progression, metabolic perturbations and toxicity (Davis, Ho *et al.* 2001). The extent of these possible side effects varies according to the type of drug, the concentration used, the type of cell and the length of exposure to the specific drug. To minimize such negative consequences of the synchronization methods, preliminary experiments were performed. Another limitation of using pharmacological synchronisation methods is that cells maintain synchrony only for one cycle after removing the drug. An alternative technique to study cell cycle dependent events was time-lapse experiments combined with microinjection of neutralizing antibodies and quantitative fluorescent microscopy (Hitomi and Stacey 2001). The major disadvantage of this method was the limited number of cells used, since the technique is based in following the behaviour of individual cells. Under these conditions, the synchronization methods are still widely used to investigate cell cycle dependent mechanisms.

5.2.2. Ras is indispensable for cell cycle re-entry and progression of quiescent cells

Previous studies demonstrated Ras importance in G0 to G1 transition through the use of anti-Ras antibodies or temperature-sensitive mutant Ras (Durkin and Whitfield 1986, Mulcahy, Smith *et al.* 1985). They further showed that introduction of hyperactivated Ras induced S phase entry in quiescent fibroblasts, whereas abrogation of endogenous Ras function in normally growing cells led to cell cycle arrest. Our ability to reproduce this established concept credits and confirms the functionality, efficiency and stability of the RasOFF heterodimerization system in a large population of cells for a relatively long duration (at least 48 hours).

We were able to demonstrate that the need for Ras activity did not stop with entry of quiescent cells in G1 phase, but was found to be prolonged until late G1. Earlier studies indicated multiple activations of Ras during G1 phase, all required for transition of serum stimulated quiescent cells in S phase (Dobrowolski, Harter *et al.* 1994, Takuwa and Takuwa 1997, Gille and Downward 1999). It was reported that Ras was active throughout G1 and reached the highest amplitude twice: in early and mid G1. However, in our case kinetic analysis of Ras protein during G0-G1 to S transition depicted a different activation profile for T98G#1#7 cells. Upon serum stimulation, these cells expressed one single peak of Ras activity in early G1 (within the first 10 minutes of mitogen stimulation). The remaining period of G1 phase was characterized by a basal and constant Ras activity. This observation suggests a cell-type dependent activation profile of Ras protein. Despite many investigations described in the literature, the possibility and precise schedule of disengagement of Ras signalling with G1 phase progression is not yet clear; and mistimed Ras activity is at the heart of tumorigenesis. In order to fill this knowledge gap, we followed cell cycle progression of mitogen-stimulated quiescent cells when Ras was periodically switched off throughout the G1 phase (every 1 hour starting from serum stimulation). We found that Ras protein, apart from regulating G1 entry, extends its control up to 5 hours after serum addition, but afterwards its signal was not required anymore. The commitment for cell cycle progression at 5 hours after stimulation suggested the presence of a possible mitogen-restriction point, a model of cell cycle control initially proposed by Pardee (Pardee 1974).

5.2.3. CyclinD1 expression is under the control of Ras in cells emerging from quiescence

Mitogen-stimulated quiescent cells' re-entry into the cell cycle is initiated by the assembly of cyclinD1-CDK4/6 complexes. The key player in transmitting mitogenic signals is Ras protein. Ras-induced cyclinD1-CDK4/6 complexes start inactivating pRB by inhibitory phosphorylation that in turn releases E2F transcription factor. Once active, E2F promotes transcription of genes responsible for cell cycle progression and S phase entry, amongst which is cyclinE. In parallel, increasing levels of cyclinD1 complexes lead to the sequestration of $p21^{CIP1}$ and $p27^{Kip1}$ which are stably bound to cyclinE-CDK2 complex. The latter is now active and can complete the inactivation of pRB that induces E2F-mediated S phase genes transcription (Filmus, Robles *et al.* 1994, Sherr 2000). In line with this regulatory mechanism of S phase entry, heterodimerizer-induced Ras inhibition in T98G#1#7 quiescent cells was associated with a downregulation of cyclinD1 expression and no hyperphosphorylation of pRB, partially explaining the block in cell cycle entry. The opposite was observed when interfering with Ras activity 6 hours after serum stimulation. Cells commitment to proliferate was accomplished regardless of the lack of mitogenic signals during the last hours of G1 phase.

Several reports demonstrated the potential of exogenously expressed, hyperactive Ras in increasing cyclinD1 production in quiescent cells lacking growth factors stimuli, thus highlighting the critical role of Raf/MEK pathway as major downstream effector of Ras (Filmus, Robles *et al.* 1994, Liu, Chao *et al.* 1995, Winston, Coats *et al.* 1996). Moreover, translation of cyclinD1 was found to be regulated, at least in part, by the PI3K/Akt pathway which in turn can be activated by Ras upon serum or growth factor stimulation (Franke, Yang *et al.* 1995, Rodriguez-Viciana, Warne *et al.* 1994). The exact mechanism of the crosstalk between these two pathways is not completely clear. However, it has been shown that NIH3T3 cell treated with a potent PI3K inhibitor (LY294002) caused a much stronger decrease in cyclinD1 expression compared to a MEK inhibitor (PD98059) (Gille and Downward 1999). The stronger effect of the PI3K inhibitor in this case is a result of inhibiting many downstream signalling pathways including serine/threonine kinase Akt/PKB, RacGTPase (part of Rho family) and mTOR pathway (Marshall 1996, White, Nicolette *et al.* 1995, Rodriguez-Viciana, Warne *et al.* 1997). In fact, Akt/PKB has been reported to induce cyclinD1 expression at mRNA level (Muise-Helmericks, Grimes *et al.* 1998) as well as it stabilizes the protein through GSK-3β (glycogen synthase kinase-3β) (Diehl, Cheng *et al.* 1998). GSK-3β-mediated

phosphorylation of cyclinD1 triggers its export from the nucleus to the cytoplasm during G1/S transition. Moreover, Ras mutants lacking the ability to interact with PI3K could not stabilize cyclinD1; while Rac GTPase was found to induce cyclinD1 expression (Westwick, Lambert *et al.* 1997). Overexpression of RacGTP was able to activate cyclinD1 promoter and transcription probably through NF-kB (Gille and Downward 1999, Joyce, Bouzahzah *et al.* 1999, Page, Li *et al.* 1999). Additionally, PI3K and Akt/PBK pathway were identified as strong activators of E2F transcription factor, even more so than Ras. These findings gave way to new ideas on how PI3k and Akt/PKB regulates E2F, for example the CDK inhibitor $p27^{Kip1}$ (Gille and Downward 1999). The involvement of many pathways is important in normal cell proliferation because it ensures at several levels the optimal conditions from the extra- and intracellular environment before the cell commits to division. In our case, the RasOFF heterodimerization system downregulated only Erk protein, while Akt activation remained the same. The persistent Akt activation, however, could not induce cyclinD1 expression in lack of Ras activity. These results gave the first hint that MAPK pathway mediates signals relayed by Ras for G0/G1/S transition, at least in T98G cells. To test if this hypothesis is correct, cell cycle analysis was performed using the MAPK/Erk and PI3K inhibitors, U0126 and LY294002 respectively, in parallel with the RasOFF system. The obtained results showed a dramatic block of cell cycle re-entry and progression from LY294002 administration prior to and few hours after serum stimulation. In contrast, the U0126 inhibitor had a milder effect, very similar to that of the heterodimerization system. Taken together, these results strongly indicate that Ras GTPase exerts its regulatory function on the cell cycle control machinery, at least in modulating cyclinD1 expression, through the MAPK/Erk pathway.

Many studies proved the influence of the Ras downstream effector Erk on several levels in regulating growth and cell cycle progression. The first demonstration that Ras-activated Erk pathway was sufficient to stimulate cyclinD1 expression was reported in the mid ‘90s (Albanese, Jonhson *et al.* 1995, Lavoie, L'Allemain *et al.* 1996). Other studies reported that cyclinD1 expression during G1 phase required continues Erk signalling (Weber, Raben *et al.* 1997b, Balmanno and Cook 1999, Roovers, Davey *et al.* 1999). Even though the exact mechanism of Erk-cyclinD1 interaction is not fully understood, there is strong evidence that members of the AP-1 proteins, including Fos and Jun, sense Erk activity through the functional docking site (DEF domains) on their C terminus and are phosphorylated by it. Once active, AP-1 proteins can induce cyclinD1 expression (Murphy, MacKeigan *et al.* 2004). Apart from

directly targeting cyclinD1 expression, Erk signalling can modulate G1 to S transition by interfering with the nucleo-cytoplasmic transport of cyclinD1 mRNA. The eukaryotic translation initiation factor E4 (eIF4E), responsible for mRNA transport (Topisirovic, Capili *et al.* 2002, Rousseau, Kaspar *et al.* 1996, Lai and Borden 2000), is phosphorylated and subsequently activated by Mnk1/Mnk2 (downstream effector of Erk) at position Ser209. Mutations of Ser209 or inhibition of Mnk1/Mnk2 resulted in downregulation of cyclinD1 (Topisirovic, Ruiz-Gutierrez *et al.* 2004). Further confirmation of the Erk-eIF4E-cyclinD1 mRNA axis, came from the lack of effect of the overexpressed eIF4E on cyclinD1 mRNA distribution (Rosenwald, Kaspar *et al.* 1995, Rousseau, Kaspar *et al.* 1996).

Another step of the G1 to S transition dependent on Erk activity is cyclinD1-CDK4/6 stabilization. This effect is mediated by the CDK inhibitor p21, which accumulates in early G1 phase upon transient activation of Erk protein (Bottazzi, Zhu *et al.* 1999, LaBaer, Garrett *et al.* 1997, Cheng, Olivier *et al.* 1999). In parallel, p27 is believed to be downregulated by Ras/Erk pathway, but the mechanism is not yet clear due to contradictory reports. In this regard, there are some reports indicating that downregulation of p27 by Erk is achieved through CDK2 and Skp2 proteins (Delmas, Manenti *et al.* 2001, Mirza, Gysin *et al.* 2004). A more recent finding is the direct interaction of Ras-activated Erk with the TSC (tuberous sclerosis complex) complex, part of the mTOR (mammalian target of rapamycin) signalling. It was shown that EGF or active Ras mutant can promote mTORC1 activity and tumorigenesis by inhibiting the GAP function of TSC. The latter is phosphorylated directly by Erk-RSK (Zoncu, Efeyan *et al.* 2011, Roux, Ballif *et al.* 2004). Although mTORC1 is activated mainly by PI3K/Akt pathway, the TSC2 (GAP TSC) has different phosphorylation sites for each signalling cascade. Additionally, Erk/RSK can induce RAPTOR (regulatory-associated protein of mTOR) phosphorylation that in turn will lead to 4E-BP activation (Pearce, Komander *et al.* 2010, Carriere, Romeo *et al.* 2011, Tang, Nunez *et al.* 1999). Carracedo *et al.* identified a negative feedback loop starting from S6K towards Akt and Erk activity (Carracedo, Ma *et al.* 2008a). Posed with several possible crosstalks between signalling pathways to modulate cell cycle progression, further investigation will be required to discriminate and identify those under the rigorous control of Ras activity.

5.2.4. Switching off Ras induces G0-G1 phase arrest in cycling cells

The indication that actively proliferating cells commit to cell cycle progression or exit to quiescence most probably in G2 rather than G1 was first described by Hitomi and Stacey in 1999 (Hitomi and Stacey 1999b). Through anti-Ras antibody microinjection in the preceding G2 phase and mitogen withdrawal as soon as mitosis is over, they demonstrated that lack of mitogen–induced Ras activity was responsible for a reduced progression into S phase of NIH3T3 cells. These observations were the starting point for several studies whose focus was exploiting G2 phase and the relevance of mitogen-induced Ras signalling. Time-lapse fluorescent microscopy investigations identified two subpopulations with clearly distinct Cdk2 activity in cells entering G1 after mitosis. Cells with low G1 cellular activity (low Cdk2 activity) were highly sensitive to mitogen withdrawal after exiting mitosis and left the cell cycle until growth factor availability was back to normal levels. On the other hand, cells possessing high G1 kinase activity were irreversibly committed to commence and culminate the cell cycle. Interestingly, sister cells had equal molecular activity at the end of mitosis, but quickly changed as cells entered G1 phase (Matson and Cook 2017). In addition, G2 Ras-induced cyclinD1 expression was stably maintained until the next S phase. Anti-Ras antibody injection as well as introduction of oncogenic Ras in G1 phase failed to alter cyclinD1 expression suggesting a Ras-dependence of cyclinD1 only in G2 phase for actively proliferating cells (Hitomi and Stacey 1999b, Sa, Hitomi *et al.* 2002).

Our investigations in asynchronous cycling cells indicated that interfering with Ras signalling did induce cell cycle arrest in G1 phase and presumably exit to quiescence. We observed an accumulation in G0G1 of T98G#1#7 cells, at the expense of S phase, within the first 24 hours of heterodimerizer-induced Ras inhibition and after 48 hours the percentage of cells in G0G1 phase increased by more than 20%. The time points measured correlate with the first and the second cell cycle completed after heterodimerizer addition, since the calculated doubling time for this cell line is around 28 hours (ATCC 2012). The progressive increase of cells blocked in G0G1 phase supports the idea that the response of cells upon activation of the RasOFF system is dependent on the stage in which the cell cycle is. Accordingly, the longer they were kept in the absence of Ras activity, the greater the number of cells blocked in G0G1.

5.2.4.1 Ras function is required in G2 and/or early G1 phase

In order to understand when Ras was relevant during cell cycle progression, we abolished Ras protein in synchronized cells in S, G2 or in the following G1 phase. Cell cycle analysis through flow cytometry of T98G#1#7 cells released from G1/S or G2/M arrest, in absence of Ras activity indicated a lower entry in the next S phase if Ras is blocked in S, G2 and in early- or mid-G1 phase. However, when measuring the percentage of cells in middle S phase (especially after the release from G2/M block) the difference between the control and treated cells had decrease. A possible explanation of these results might be that inhibition of Ras activity in the preceding G2 phase and/or until the middle of the following G1 phase can prolong progression of cells through G1 and consequently delay S phase entry. Western blot analysis of cells released from the G2/M arrest in absence of Ras activity demonstrated a downregulation of cyclinD1 expression. As expected, no difference was seen if Ras inhibition occurred in mid/late G1 phase. Further investigations are required in order to identify if Ras is necessary only in the preceding G2 phase as it is supported by previous studies (Hitomi and Stacey 1999a, Hitomi and Stacey 1999b, Sa, Hitomi *et al.* 2002) or perhaps in early G1 as well. Looking at the activation of the two major downstream effectors of Ras, only Erk was downregulated reflecting Ras activity and placing it as the mediator of Ras signalling to the regulatory cell cycle machinery. However, the implication of Ras/Erk pathway in modulating G2/M progression has been continuously questioned due to inconsistent reports, especially regarding Erk activity. On one hand, several groups demonstrated an active Erk that if inhibited, delayed G2/M transition and cell cycle progression (Liu, Yan *et al.* 2004, Wright, Munar *et al.* 1999). On the other hand, it was demonstrated that MEK activity was more important than Erk (Harding, Giles *et al.* 2003).

The common feature observed during the investigations in actively cycling cells and those emerging from quiescence was Ras-independent Akt activity. Even though PI3K/Akt pathway is considered the second best characterized downstream effectors of Ras, exceptions exist. The cell line used in our study for cell cycle progression analysis is the T98G derived from glioblastoma multiforme. One of the characteristics of this aggressive cancer is the overexpression of epidermal growth factor receptors (EGFR) that increases the possibilities of a hyperactivation of Ras/MEK/Erk pathway (Houillier, Lejeune *et al.* 2006, Ohgaki, Dessen *et al.* 2004). In addition, other reports demonstrated that to maintain some of the properties of

this type of cancer PI3K/Akt pathway is of great importance (Sunayama, Sato *et al.* 2010a). Moreover, it was shown that the existence of a negative feedback that links these two signalling cascades where inhibition of either one of them promotes the activation of the other (Sunayama, Matsuda *et al.* 2010). This crosstalk might be a possible explanation of the inability of our system to block, at least partly, Akt phosphorylation levels. On the other hand, it has been reported that hyperactivation of both Ras/Erk and PI3K/Akt pathways are necessary in cancer formation but at a later moment PI3K/Akt can take over and Ras signalling is no longer required (Lim and Counter 2005).

5.2.4.2. An alternate cell-model to investigate Ras-dependent cyclinD1 expression

HeLa#1#7 cells were also used to evaluate Ras function in actively cycling cells. Inhibition of Ras activity in mid G1 phase did not impair cyclinD1 expression. Even though the results support what we observed in T98G cells, further use of HeLa cells in investigating G1-to-S transition is not possible since they have an impaired G1 checkpoint, thus they continue proliferating. This is due to the infection with human papillomavirus (HPV) which is accompanied by two viral oncogene E6 and E7 (zur Hausen 1999). Expression of E6 and E7 proteins negatively influences the cell cycle control by inhibiting the normal function of p53 and pRB, respectively. E6 protein was shown to bind and mediate the degradation of p53 tumour suppressor protein, while E7 disrupted pRB/E2F complexes by permanently binding to pRB (Mantovani and Banks 2001, Munger, Basile *et al.* 2001).

Moreover, Akt protein showed a different phosphorylation profile in HeLa cells compared to the glioblastoma T98G cells. The shift of the phosphorylation site (from Thr308 to Ser473) in cells released from mitosis can be due to sequential phosphorylation process of Akt in order to achieve its maximal activation. A second possibility is the detection of three distinct bands that correspond to the three Akt isoforms (Akt1, Akt2 and Akt3). The expression and function of each isoform within the cells is not yet clear due to controversial findings (Wee and Wang 2017). We tried to discriminate between the three isoforms using antibodies that specifically recognized one of the Akt proteins. Some preliminary data showed Akt1 being phosphorylated at Thr308 and Akt2 at Ser473. However, detection of the total Akt indicated a higher expression of Akt1 in comparison to the other isoforms (data not shown).

Another surprising event that we encountered while working with HeLa cells was the lack of Akt inhibition from the PI3K inhibitor, LY294002. The latter is a potent inhibitor of PI3K/Akt but exerts its function in a dose- and time- dependent manner. LY294002 is reported to induce apoptosis if used in high concentration (30-50µM) and for long periods of time (more than 24 hours). There is evidence that the response to lower doses of this inhibitor (lower than 30µM) is cell-type specific. Jo *et al.* show a similar Akt activity in HeLa cells, where treatment with 20M LY294002 for 14 hours did not abolish Akt phosphorylation (Jo, Lo *et al.* 2011). Other studies found that incubation of HCC cells (hepatocellular carcinoma) with 25µM LY294002 for 48 hours induced a progressive decrease of Akt phosphorylation leading only to G1 cell cycle arrest and no induction of apoptosis (Kunter, Erdal *et al.* 2014). The opposite effect was seen upon treatment with 50µM LY294002 for 24 hours in four out of five different human primary effusion lymphoma cell lines tested (Uddin, Hussain *et al.* 2005).

To summarize, our investigation of the functional relevance of Ras in the control of cell cycle progression demonstrated its absolute requirement in early G1 phase for both quiescent and actively proliferating cells. However, it must be noted that our results in cycling cells cannot exclude the possibility that G2 phase Ras activity may take precedence in the commitment to the ensuing cycle, as is widely believed in the field. Carefully designed further studies are needed to clarify whether Ras activity in G2 is alone sufficient to determine entry into the next round of cell cycle or whether Ras function is also essential through the post-mitotic G1-phase in continuously proliferating cells. Our results complement current-day understanding of Ras signalling by clearly demonstrating that cell cycle re-entry of quiescent cells is less dependent on isolated peaks of Ras activity in G1 phase, but rather relies heavily on sustained Ras signalling until mid-G1, that drives cyclinD1 expression. Investigations into the three major effector arms downstream of Ras (Erk, PI3K and RalA), excluded PI3K and RalA as mediators of Ras's cell cycle effects, due to their sustained activity even in absence of Ras signalling. It is noteworthy that our data do not support the phenotype documented in Rasless MEFs where cyclinD1 was found to be normally expressed, although inactive. Repression of Ras activity using the RasOFF system induced downregulation of cyclinD1 and retained the pocket protein pRB in its hypophosphorylated form, thus leading to a cell cycle arrest in quiescence. Taken together our results portray Erk as the only acutely Ras-responsive

signalling mediator, at least with regard to the control of cell division, particularly responsible for cyclinD1 expression in G1 of cycling cells and of cells emerging from quiescence.

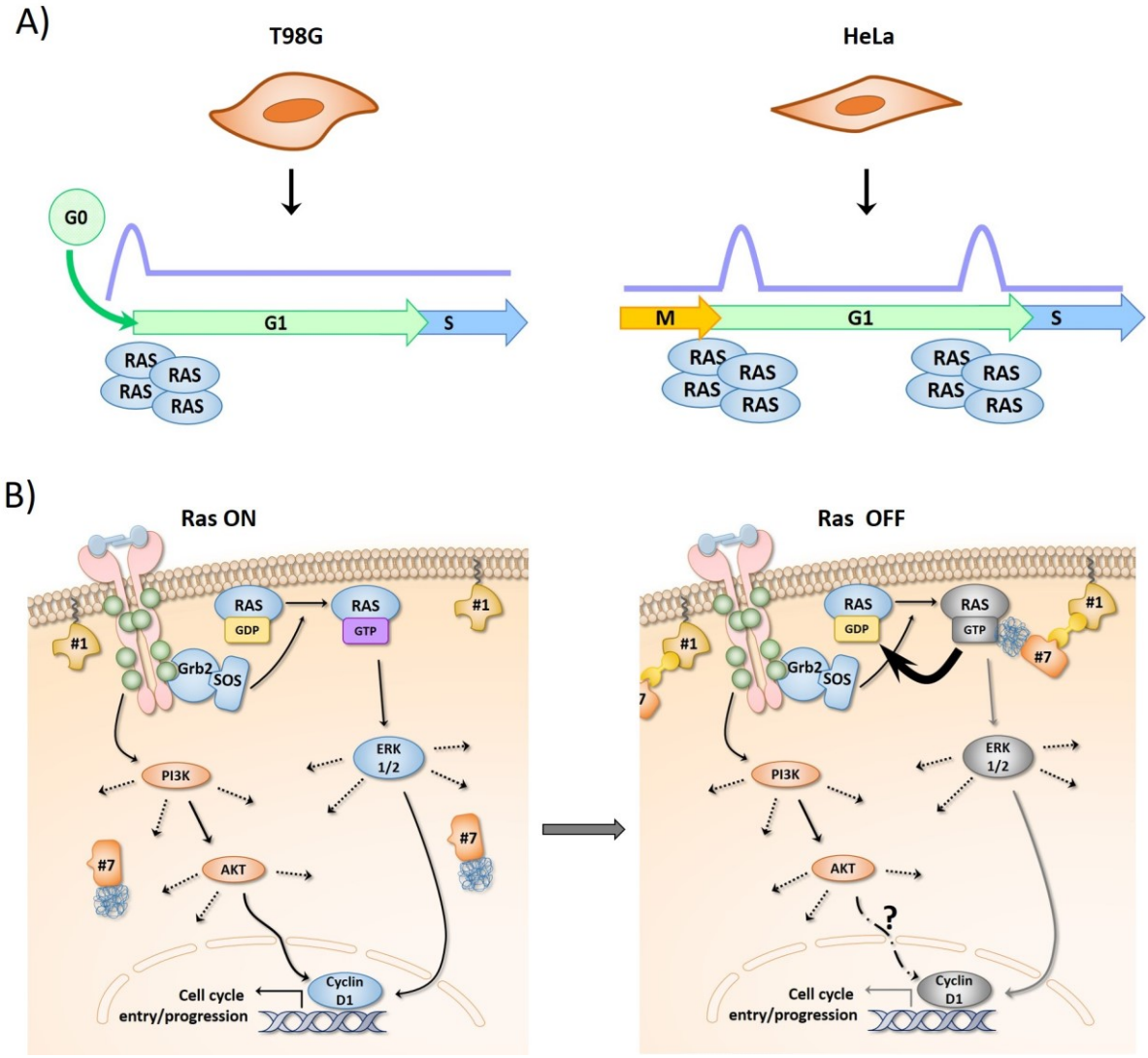

Figure 36. Model summarizing Ras activity and involvement in the cell cycle progression.

A) Cell-type dependent activation profile of Ras protein. Upon serum stimulation, quiescent T98G cells express one single peak of Ras activity in early G1, while the remaining period of G1 phase is characterized by a basal and constant Ras activity. Dissection of G1 phase demonstrated that G0-G1 to S phase progression is Ras-dependent up to 5 hours after serum stimulation of quiescent cells. HeLa cells, instead, experienced two genuine peaks of Ras activity in early and late G1 phase, respectively. B) Inactivation of Ras protein induced downregulation of cyclinD1 probably using Erk as transmitter of its signalling. PI3K pathway, a second cascade believed to control cyclinD1, could not induce its expression even though constantly active.

6. Conclusions and future perspectives

In this thesis, we present a new experimental technique able to directly and acutely inhibit one of the most studied membrane-bound small GTPases – Ras. We have demonstrated that the RasOFF heterodimerization system can directly and specifically decrease Ras activity without affecting other signalling pathways, including other small GTPases. Furthermore, we were able to prove that in the absence of the heterodimerizer, the presence of the two fusion proteins did not alter cellular behaviour; and the heterodimerizer itself showed no effect on cells lacking fusion proteins.

At the experimental level, it is important to emphasize the efficacy and applicability of the RasOFF system for studying Ras-dependent proliferation of entire cell populations. Most of the studies that described the correlation between mitogen-induced Ras activity and cell proliferation were performed in single cells, which despite the optimal experimental conditions are still not the best representation of what happens under physiological conditions. A second advantage of our technique is the simplicity of the system, in that Ras inhibition is induced just by the simple addition of an inert, membrane-diffusible heterodimerizer to the culture media, as opposed to the harsh antibody microinjection method. Last but not least, the RasOFF system can be activated at any desirable time and rapidly affects Ras function within minutes.

By using the RasOFF heterodimerizer system, we investigated the necessity of Ras in the overall control of cell cycle. Our data decisively showed that Ras signalling is obligatory for quiescent cells to re-enter and progress through G1 phase. We further established that the requirement for Ras activity in cells emerging from quiescence extends up to 5 hours after mitogen stimulation. On the other hand, actively proliferating cells displayed a stronger Ras activity profile during G2 phase followed by a decline as cells progressed through mitosis and G1 phase. We were able to demonstrate that S phase entry is controlled by Ras activity via regulating cyclinD1 expression in cells emerging from quiescence and actively cycling cells. Pharmacological inhibitor studies combined with the RasOFF system indicated that Ras

mediates its cell cycle effects via Erk. Strikingly, Akt activity was entirely uncoupled from Ras in this context.

In the near future, our work will be extended to delineate the molecular effectors downstream of Ras/Erk, that transmit their signals from the cytosol to the nuclear cell cycle control machinery. Further analyses are required to clarify contextual relevance of Ras signalling during the G1 phase of actively cycling cells.

In the long term, the RasOFF system will be employed to investigate importance of Ras in inflammation. This will be achieved by introducing the heterodimerization system in immune cells by way of creation of a conditional mouse model.

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Ehrenwörtliche Erklärung

Hiermit erkläre ich ehrenwörtlich im Zusammenhang mit der Beantragung der Eröffnung meines Promotionsverfahrens:

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3. dass mich folgende Personen bei der Auswahl und Auswertung des Materials unterstützt haben: PD Dr. I.Rubio, Dr. Helmut Pospiech und Yvonne Schlenker

4. dass die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

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6. dass ich nicht eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, den 11.12.2017 Unterschrift des Verfassers

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